utes after addition of 0.2 ml of 0.02 M $CaCl_2$ to 0.2 ml of plasma. Clot formation was not apparent 4 hours after $CaCl_2$ had been added to plasma from the tricarballylate incubated blood samples.

Discussion. These experiments indicate that blood clotting is inhibited *in vitro* in the presence of isocitrate and *cis*-aconitate as well as in the presence of citrate. None of the dicarboxylic acids tested was effective in prolonging clotting time; the lactone of isocitric acid was similarly ineffective. The mechanism of the inhibition has not been established. Although other interpretations are possible, it is likely that the tricarboxylic acids chelate with ionic calcium through the 2 terminal carboxyl groups as postulated by Heinz(4) to form relatively undissociable calcium complexes in the blood. Tricarballylate has been shown to complex with ionic calcium in pure solutions(5), but it appears from the recalcification experiments that its ability to prevent clotting stems from interference with the coagulation mechanism other than its Ca^{++} binding ability.

In the normal state it is unlikely that *cis*-aconitate or isocitrate play a significant role in prevention of blood clotting *in vivo* because of the small concentration of these substances in blood(6). It is possible, however, that these intermediates of the Krebs cycle might influence the intracellular concentration of ionized calcium. This concept would be especially important in those tissues, such as nerve and muscle, where small changes in concentration of ionized calcium are known to exert profound effects(7). Conversely, increased concentrations of ionized calcium in tissues might influence citric acid cycle metabolism by complexing with citrate, isocitrate or *cis*-aconitate thereby interfering with enzyme substrate formation.

Summary. Human blood was incubated *in vitro* with the sodium salts of a number of di- and tricarboxylic acids and their effect on clotting time was noted. *Cis*-aconitate, *trans*-aconitate, isocitrate and tricarballylate markedly increased clotting time of whole blood. Prolongation of clotting in the presence of isocitrate and *cis*-aconitate was dependent upon their concentration in blood. The possible mode of action of these metabolically active compounds and the implications of the findings were discussed.

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Role of Relaxin in Stimulating Mammary Gland Growth in Mice.* (24293)

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It has been shown that estrogens (E) will stimulate mammary gland duct growth while E and progesterone (P) in suitable amounts and ratios will stimulate lobule-alveolar growth in normal and gonadectomized male and female animals(1-6). It has been suggested that relaxin synergizes with E and P in stimulating lobule-alveolar growth in rats

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(7,8) and potentiates estrogen in guinea pigs and rabbits(9), but is of little value in mice (10). Using mammary gland spreading factor as an index of mammary gland growth in rats, relaxin and E stimulated a large increase of spreading factor, but relaxin was of no benefit as an addition to combination of E and P. Relaxin alone failed to stimulate lobule-alveolar growth(11-13). In analyzing the variable observations on the role of relaxin in stimulating mammary lobule-alveolar growth, it was noted that relaxin was most effective when less than optimal amounts of P were administered. In fact, E and relaxin alone seemed to be effective(8,13). These observations could all be harmonized by the theory that injection of E and P stimulates endogenous production of relaxin. Relaxin thus stimulated acts upon the pituitary to stimulate mammogen secretion, resulting in mammary lobule-alveolar growth. If this is true, then E and relaxin should be as effective as E and P in stimulating mammary gland growth. Total desoxyribosenucleic acid (DNA) of the mammary gland, shown to be an improvement in quantitative estimation of lobule-alveolar growth(5,14,15,16) has been used in the present study to substantiate this hypothesis.

Materials and methods. Since relaxin is obtained from sow ovaries, tests were conducted to insure freedom from progesterone contamination in relaxin preparations used. None was found, but all relaxin‡ preparations were routinely washed with ether (anhydrous) several times as a safeguard. Male albino mice (intact and castrated) weighing 16-18 g were used. To their feed was added 1.23 mg/kg of diethylstilbestrol for approximately 4 weeks to stimulate extensive duct system. Suspensions of relaxin alone and relaxin in combination with estradiol benzoate in 0.1 ml of olive oil were then administered subcutaneously daily for 10 days. On 11th day, mammary glands were removed from each mouse killed by excessive ether anesthesia, and ¾ of glands were used for DNA determination(15) and ¼ reserved for examination of whole mounts. After diethylstilbestrol pretreatment, one group of mice was hypophysectomized, then divided into 2 lots, one receiving E alone and the other E and relaxin for 10 days.

Results. In previous work it was shown that total DNA content of male mice glands after 4 weeks of diethylstilbestrol pretreatment to induce duct growth averaged 1.51 mg ± 0.56 mg. Injection of 0.75 μg estradiol benzoate and 0.75 mg progesterone daily for 10 days increased total DNA of the glands to 2.28 mg ± 0.275 mg representing growth of the lobule-alveolar system(15).

When the same level of estradiol benzoate (0.75 μg) was injected with graded levels of relaxin in both normal and castrated male mice, there was an increase in average total DNA (Table I). Optimal level of relaxin appears to be 2.5 guinea pig units (GPU). Injection of E alone failed to stimulate an increase in total DNA in intact animals, but induced a slight increase in total DNA in the castrated animals. When relaxin alone was injected in graded amounts total DNA remained stationary throughout entire dosage range, though it was clearly higher than those injected with olive oil.

Examination of whole mounts of the mammary glands revealed extensive growth of the lobule-alveolar system in mice receiving E and relaxin, whereas those receiving either E or relaxin, but not both, showed only duct growth. The extent of lobule-alveolar growth in various groups was roughly proportional to their DNA content. Intact animals injected with E and 2.5 GPU relaxin showed the best development of lobule-alveolar system, and mammary gland growth of some animals in this group was equivalent to that at 8 days of pregnancy(17).

Castrated mice injected with relaxin alone showed no alveolar growth, whereas those injected with E showed very slight lobule-alveolar growth in some cases. With E and 2.5 GPU relaxin best alveolar growth was observed. While intact animals showed slightly greater total DNA, the possible influence of androgen secretion will require further study.

‡ Relaxin preparation, 1164A-Lot 53, kindly supplied by Dr. R. L. Kroc, Chilcott Lab., Morris Plains, N. J., assayed 30 GPU/mg.

TABLE I. Effect of Estrogen and Relaxin on Mammary Alveolar Growth in the Mouse.

Treatment (amt/day)		No. of mice	Total DNA Mean (mg)	S.E.	DNA/mg D.F.F.T.* Mean (μg)	S.E.
		Intact male				
Control		10	1.369	.088	33.5	1.4
Olive oil	.1 ml	9	1.347	.130	31.9	1.5
Olive oil +						
Estradiol benzoate	.75 μg	23	1.572	.047	31.5	1.0
E.B. + relaxin	.62 GPU	11	1.937	.091‡	37.4	1.4
Idem	1.25 "	16	1.999	.119‡	38.6	1.4
"	2.50 "	18	2.258	.095‡	39.3	1.2
"	5.00 "	16	2.019	.161†	39.4	1.9
Olive oil + relaxin	1.25 "	8	1.775	.139§	36.6	2.3
Idem	2.50 "	9	1.713	.132	42.2	2.2
"	5.00 "	32	1.737	.074§	34.8	3.3
"	10.00 "	9	1.626	.076	32.3	1.3
Olive oil + E.B. + P	.75 mg	5	2.278	.275	26.5	5.0‖
		Castrate male				
Olive oil +						
Estradiol benzoate	.75 μg	7	1.808	.106	26.9	1.5
E.B. + relaxin	.62 GPU	5	1.787	.078	28.7	1.7
Idem	1.25 "	7	1.797	.158	29.6	1.4
"	2.50 "	6	2.144	.142	31.3	2.2
"	5.00 "	7	1.858	.143	32.0	1.3
Olive oil + relaxin	5.00 "	7	1.645	.072	32.2	.8
		Hypophysectomized male				
Olive oil +						
Estradiol benzoate	.75 μg	6	1.477	.089	42.9	4.2
E.B. + relaxin	2.50 GPU	6	1.708	.071	43.2	2.6

* D.F.F.T. = Dry, fat-free tissue. † Significant at 1% level when compared to group inj. estradiol benzoate .75 μg. ‡ Significant at .1% level. § Significant at 5% level when compared to group inj. olive oil alone. ‖ Data from Damm and Turner(15).

Hypophysectomized male mice treated with E showed evidence of slight regression in total DNA. When E and 2.5 GPU of relaxin were administered, total DNA equaled that of E in castrate mice. No lobule-alveolar growth was stimulated in these animals. Hypophysectomized animals injected with combination of E and relaxin were somewhat more vigorous than those injected with E alone in the later half of injection period, and the duct system of the former seemed to be more normal in comparison to involuted (shriveled) condition of the latter.

Discussion. The present study shows that E and relaxin in optimal amounts stimulate growth of the mammary lobule-alveolar system of intact and castrate male mice equal to that obtained by injection of E and P for equal periods(15). Two indices of growth were employed, namely, total DNA of the glands and visual observation of whole mounts to determine type and extent of gland growth. In absence of the pituitary, E and relaxin are without effect upon lobule-alveolar growth. These observations confirm previous reports that ovarian hormone-relaxin combinations are ineffective in hypophysectomized animals (7,8). Since relaxin is not effective in hypophysectomized animals, it is not a substitute for pituitary mammogen.

It has been suggested that P acts upon the anterior pituitary to stimulate increased secretion of mammogen(18). Present data suggest that relaxin may act in this way and thereby stimulate lobule-alveolar growth.

As a tentative theory, it is suggested that normal growth of the lobule-alveolar system during pregnancy is dependent upon the following sequence of hormone action. E and P together stimulate the endogenous secretion of relaxin. Previous work(19) suggested that E alone has a direct local action upon the mammary gland area in increasing hyperemia and permeability of the vascular system,

thereby increasing circulating nutrients and hormones available to the mammary gland.

Relaxin rather than P acts upon the anterior pituitary to stimulate increased secretion of mammogen. This theory would assign P to an indirect role in mammary gland growth although it is possible that P and relaxin are equally effective at the pituitary level.

The hormones and glands involved in endogenous relaxin secretion become of paramount importance if relaxin rather than P stimulates pituitary mammogen secretion and lobule alveolar growth. Since the growth of the mammary gland of male animals is stimulated by E and P, it would follow that relaxin may be secreted in the male although, to date, relaxin has not been detected in male animal blood following injection of E and P.

Summary. 1) Using DNA as index of mammary gland lobule-alveolar growth in intact and castrate male mice, it was shown that daily injection of 0.75 μg estradiol benzoate and 2.5 GPU of relaxin for 10 days stimulated increases in DNA equal to that produced by the same level of estrogen and 0.75 mg progesterone. Therefore, relaxin no longer need be considered merely a synergist of estrogen and progesterone in mammary gland growth. 2) Estrogen and relaxin at above levels were without effect upon mammary alveolar growth in hypophysectomized mice. Relaxin, therefore, is not comparable to pituitary mammogen as a stimulator of mammary gland growth. 3) It is suggested estrogen and progesterone stimulate endogenous secretion of relaxin and relaxin, in turn, stimulates increased secretion of mammogen by the pituitary.

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Surface Activation of Plasma Clotting: a Function of Hageman Factor.* (24294)

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The influence of surface contact on blood clotting has been recognized since the observations of Hewson (1770), Lister (1863), and Freund (1886). Modern literature contains many experiments which have attempted to implicate one or other of the clotting factors. Previously, no one explanation of the effect seemed adequate. Recent evidence suggests that Hageman factor may have a role in surface phenomena(1,2,3). In this communica-

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