special technical precautions. This may account for the lack of reports of signs or symptoms in man or animals upon return to sea level following extended periods in 100% oxygen at reduced pressures or He-O_2 mixtures(2,6,11,14,15), assuming that denitrogenation *via* 100% O_2 or He-O_2 produces the same effect. Nevertheless, it remains a matter of some interest that N_2 may exert any depressant effect at all at sea level pressures. If in fact we are dealing with an aspect of N_2 narcosis, the increased sensitivity to N_2 should show up much more dramatically, as the observation of Bond(3) would imply, if the first exposure to N_2 were to take place at partial pressures higher than those at sea level.

Summary. Mice, rats and chicks were maintained in a 79% He-21% O_2 environment for 10-22 days. When measured in He-O_2, their metabolism was higher than that of controls maintained and measured in air. On transfer to 100% O_2, metabolism of the controls remained relatively unchanged, but that of experimentals fell to control levels. On first exposure to air, the animals maintained in He-O_2 showed a significant 7-11% depression in O_2 consumption below controls. The suggestion is made that the period of denitrogenation preceding the measurements may have sensitized the He-O_2 animals to the depressant properties of N_2.

We thank Susie Kreglow and Joe Pitt for technical assistance.

1. Behnke, A. R., Yarbrough, O. D., Am. J. Physiol., 1939, v126, 694.
2. Bond, G. F., Arch. Environl. Health, 1964, v9, 310.
3. ———, ibid., 1966, v12, 85.
4. Cook, J. F., J. Cell. & Comp. Physiol., 1950, v36, 115.
5. Cook, S. F., South, S. E., Am. J. Physiol., 1953, v173, 542.
6. Dines, J. H., Hiatt, E. P., J. Appl. Physiol., 1964, v19, 17.
7. Froese, G., ibid., 1960, v15, 53.
8. Haldi, J., ibid., 1961, v16, 923.
9. Hodgman, C. D., Handbook of Physics and Chemistry, Chemical Rubber 44 ed., 1963.
10. Leon, H. E., Cook, S. F., Am. J. Physiol., 1960, v199, 243.
11. MacHattie, L., Rahn, H., Proc. Soc. Exp. Biol. and Med., 1960, v104, 772.
12. Rhoades, R. A., Weiss, H. S., Wright, R. A., J. Lab. Clin. Med., in press.
13. Rhoades, R. A., Weiss, H. S., Wright, R. A., Hiatt, E. P., Fed. Proc., 1965, v24, 215.
14. Robertson, W. G., Hargraves, J. H., Halucher, J. E., Welch, B. E., Aerospace Med., 1964, v35, 618.
15. Robertson, W. G., Zeft, H. J., Behar, V. S., Welch, B. E., ibid., 1966, v37, 457.
16. Schreiner, H. R., Gregorie, R. C., Lawrie, J. A., Science, 1960, v136, 653.
17. Weiss, H. S., Wright, R. A., Hiatt, E. P., Aerospace Med., 1965, v36, 201.
18. Wright, R. A., Lessler, M. S., Weiss, H. S., Hiatt, E. P. ibid., 1965, v36, 201.

Received August 25, 1966. P.S.E.B.M., 1967, v124.

Influence of Growth Hormone, Steroids and Relaxin on Acid Phosphatase Activity of Connective Tissue. (31695)

B. G. STEINETZ AND J. P. MANNING (Introduced by A. Meli)
Department of Physiology, Warner-Lambert Research Institute, Morris Plains, N.J.

The pubic joint of the mouse offers an interesting model for the study of hormonal influences upon connective tissue. At this site cartilage may be transformed to ligamentous connective tissue under the influence of estrogens and relaxin(1, for review). This process, which is accompanied by "depolymerization" of acid mucopolysaccharides of the ground substance(2) and erosion of pubic bone by osteoclasts(3) has been termed "pubic symphyseal relaxation". The process is of interest not only because it represents a reversion from an adult to a more embryonic form of connective tissue but because many hormones have been implicated in the reversible transformations which occur. Thus,

thyroid and growth hormone favor(4-8), whereas glucocorticoids, androgens and progestogens oppose, pubic symphyseal relaxation(9). Crelin(3) postulated that "catalytic" agents released from the cartilage cells were responsible for the transformation of cartilaginous to ligamentous connective tissue. Others(8,10) have extended this hypothesis and suggested that estrogens activate or induce catabolic enzyme systems in both the bony and cartilaginous elements and that such enzymes act to reverse the calcification process, promote water imbibition and remove interfibrillary ground substance. Using histochemically determined acid phosphatase as an indicator of lysosomal particles (11,12), Manning *et al*(10) showed that treatment of ovariectomized mice with an estrogen greatly enhanced the activity of this enzyme in osteoclasts and chondroclasts in the pubic joint. However, acid phosphatase activity so determined was not perceptibly influenced by addition of relaxin or progesterone (10). Recognizing the shortcomings of the quantitative aspects of histochemistry, we have now repeated and extended the previous study(10) using a precise biochemical determination for acid phosphatase activity(13). In addition to the effects of estrogen, relaxin and progesterone on the acid phosphatase of the pubic symphysis of the ovariectomized mouse, we have studied the influence of growth hormone on the enzymatic response to estrogen and relaxin in the hypophysectomized mouse.

Materials and methods. One hundred twenty-seven ovariectomized and 72 hypophysectomized* female mice (CD-1) obtained from Charles River Breeding Laboratories were studied. The animals were obtained at a weight of 20-22 g and maintained in our laboratory for approximately 3 weeks before being placed on experiment. The mice were then administered hormones in the various combinations shown in Table I. Preparations were injected subcutaneously as follows:

1. ECP (estradiol cyclopentylpropionate, Upjohn, 5 μg/0.2 ml corn oil day 0.
2. GH (Growth Hormone, NIH-GH-B7), 0.5 mg/0.2 ml alkaline saline daily for 7 days.
3. R (Relaxin, Warner-Lambert W1164, 48E-2103a), 1μg = 1 unit/0.2 ml 1% benzopurpurine 4B on day 7.
4. P(Progesterone, U.S.P.), 1 mg/0.2 ml sesame oil on day 7.
5. Vehicle. Approximately half of each group of mice not receiving relaxin was injected with 0.2 ml 1% benzopurpurine 4B on day 7. Since this treatment did not influence acid phosphatase, the vehicle-treated and untreated animals were grouped together.

All mice were killed on day 8 of the experiment, the pubic symphysis exposed and interpubic ligament length measured(14). Acid phosphatase was determined according to the method of Manning *et al*(13) after homogenizing the excised pubic joint in ice cold water with a motor driven Tenbroeck grinder. Preliminary experiments established that the interpubic ligaments themselves were devoid of enzymatic activity in agreement with previous histochemical findings(10). Accordingly, these were removed in groups 3, 5 and 13 prior to analysis of the remaining connective tissues. The samples analyzed were obtained by making a saggital cut through the pubic bones along the medial border of each obturator foramen. The homogenates were thus comprised of the angles of the pubic bones, hyaline and fibro-cartilage and some surrounding connective tissue, and weighed 3-6 mg. Samples were kept as uniform as possible by a) one operator performing all of the dissections, b) inspection of each preparation at 13 $\times$ magnification.

Body weights were recorded but are not presented because the changes were entirely similar to those observed in an earlier study (7).

Results are tabulated as mean values $\pm$ SE $\bar{m}$ and significance of differences are determined according to the "T" test of Student.

Results. Ovariectomized mice. A single injection of 5 μg ECP (Group 2) significantly increased pubic symphyseal acid phosphatase over values observed in untreated ovariectomized mice (Group 1) when measured 8 days later (Table I). A further significant in-

* Criteria for completeness of hypophysectomy were similar to those previously employed (7,8).

crease was observed when 1 μg R was injected 7 days after the estrogen and 24 hours prior to autopsy (Group 3, Table I). R alone (Group 7) did not stimulate phosphatase activity. A 1 mg dose of P administered day 7 did not alone stimulate acid phosphatase activity (Group 6) nor modify the effect of ECP (Group 4), although there was some indication that it reduced the stimulation produced by ECP plus R together (compare Groups 3 and 5).

Hypophysectomized mice. Pubic symphyseal acid phosphatase activity was lower in hypophysectomized (Group 8) than in ovariectomized (Group 1) mice (Table I). Furthermore, treatment of hypophysectomized mice with ECP alone (Group 9) or GH (Group 11) alone only restored the level of enzymatic activity to that seen in untreated ovariectomized mice (Group 1, Table I). Also, addition of R to the ECP treatment (Group 10) did not increase the phosphatase

TABLE I. Influence of Hormones on Acid Phosphatase Activity of Pubic Joints of Mice.

Operation	Group	Treatment*: ECP, 5 μg (day 0)	R, 1 μg (day 7)	P, 1 mg (day 7)	GH, 0.5 mg (days 0-7)	No. mice	Acid phosphatase activity, units/mg†	Interpubic distance, mm†
Ovariex	1	0	0	0	—	27	14.9 ± .86	.18 ± .01
	2	+	0	0	—	29	23.8 ± 1.27	.73 ± .05
	3	+	+	0	—	16	39.1 ± 2.06	2.67 ± .25
	4	+	0	+	—	18	21.2 ± 1.60	.70 ± .10
	5	+	+	+	—	18	32.4 ± 2.05	1.22 ± .10
	6	0	0	+	—	11	15.6 ± 1.44	.17 ± .02
	7	0	+	0	—	8	12.4 ± 2.81	.21 ± .02
Hypox	8	0	0	—	0	10	5.3 ± .74	.11 ± .01
	9	+	0	—	0	10	13.5 ± 1.84	.23 ± .04
	10	+	+	—	0	11	12.3 ± 1.72	.52 ± .09
	11	0	0	—	+	14	12.8 ± 1.13	.21 ± .02
	12	+	0	—	+	13	23.5 ± 1.61	.54 ± .07
	13	+	+	—	+	14	32.8 ± 3.13	3.16 ± .28

P values for phosphatase

Group	1	2	3	4	5	6	7	8	9	10	11	12	13
1	—	<.01	<.01	<.01	<.01	NS	NS	<.01	NS	NS	NS	<.01	<.01
2		—	<.01	NS	<0.1	<.01	<.01	<.01	<.01	<.01	<.01	NS	<.02
3			—	<.01	<.05	<.01	<.01	<.01	<.01	<.01	<.01	<.01	NS
4				—	<.01	<.02	<.02	<.01	<.01	<.01	<.01	NS	<.01
5					—	<.01	<.01	<.01	<.01	<.01	<.01	<.01	NS
6						—	NS	<.01	NS	NS	NS	<.01	<.01
7							—	<.02	NS	NS	NS	<.01	<.01
8								—	<.01	<.01	<.01	<.01	<.01
9									—	NS	NS	<.01	<.01
10										—	NS	<.01	<.01
11											—	<.01	<.01
12												—	<.02

P values for interpubic distance

Group	1	2	3	4	5	6	7	8	9	10	11	12	13
1	—	<.01	<.01	<.01	<.01	NS	NS	<.01	NS	<.01	NS	<.01	<.01
2		—	<.01	NS	<0.1	<.01	<.01	<.01	<.01	NS	<.01	<.05	<.01
3			—	<.01	<.01	<.01	<.01	<.01	<.01	<.01	<.01	<.01	NS
4				—	<.01	<.01	<.01	<.01	<.01	NS	<.01	NS	<.01
5					—	<.01	<.01	<.01	<.01	<.01	<.01	<.01	<.01
6						—	NS	<.02	NS	<.01	NS	<.01	<.01
7							—	<.01	NS	<.01	NS	<.01	<.01
8								—	<.01	<.01	<.05	<.01	<.01
9									—	<.05	NS	<.01	<.01
10										—	<.05	NS	<.01
11											—	<.01	<.01
12												—	<.01

* All mice killed on day 8 of experiment.
† ± S.E.

activity above that observed with ECP alone (Group 9). However, combined treatment of hypophysectomized mice with ECP and GH (Group 12) resulted in an acid phosphatase activity similar to that observed with ECP alone in ovariectomized mice (Group 2). A combination of GH, ECP and R in hypophysectomized mice (Group 13) resulted in pubic symphyseal acid phosphatase levels which were significantly higher than those obtained with ECP and R (Group 10) or ECP and GH (Group 12), and did not differ significantly from those observed in ovariectomized mice treated only with ECP plus R (Group 3) although the average value was somewhat lower.

Interpubic ligaments. Interpubic ligament lengths are shown in Table I. In conformity with previous studies (4-7, 9, 14) ECP increased the interpubic gap in ovariectomized mice and synergized R in inducing formation of long interpubic ligaments in such animals. The effect of ECP and R was antagonized by concomitant administration of P, while R or P were without effect when administered singly. In hypophysectomized mice, neither ECP nor ECP plus R had a significant influence on pubic separation and GH was required to restore the response to normal (7,8).

Discussion. Present studies show a close relationship between pubic symphyseal acid phosphatase activity and the consequences of estrogen priming and administration of relaxin.

Pubic symphyses of unprimed ovariectomized mice had an acid phosphatase level of 14.9 $\pm$ 0.86 units/mg. This value was lower than we have observed in intact untreated mice (21.7 $\pm$ 1.07 units/mg; Manning, unpublished data) although interpubic distance was similar in ovariectomized and intact mice (0.18 *vs.* 0.20 mm). Administration of R alone to ovariectomized mice failed to influence acid phosphatase, whereas a single dose of 5 μg ECP increased enzymatic activity to 23.8 $\pm$ 1.27 units/mg (a value similar to that found in intact mice). When R was injected following ECP priming of ovariectomized mice, acid phosphatase levels rose to 39.1 $\pm$ 2.06 units/mg and this change was accompanied by the formation of long interpubic ligaments. Pubic symphyseal acid phosphatase was very low (5.3 units/mg) in hypophysectomized mice, and ECP treatment only restored the enzyme activity to the level seen in ovariectomized control mice. R failed to synergize ECP in hypophysectomized mice. GH treatment increased the acid phosphatase levels of hypophysectomized mice to those observed in ovariectomized mice. GH also restored the phosphatase responses (as well as the morphological responses of the pubic symphysis) to ECP and to ECP plus R in hypophysectomized mice. These data strongly suggest that hypophyseal GH (or similar hormone) normally not only maintains this enzyme even in the absence of the ovaries, but also participates in the priming action of estrogens on the pubic symphysis. In view of recent findings in man(15) it is also possible that one function of estrogen priming itself is the induction of increased secretion of hypophyseal factors similar to GH, although this has not been established in mice.

When ovariectomized mice are treated with estrogens, the multinuclear cells of the pubic symphysis (osteoclasts, chondroclasts and free giant cells) become hyperplastic(3,8), and histochemically-determined acid phosphatase activity is intensified in these cells as well as in osteocytes and chondrocytes(10). Addition of R failed to influence the staining intensity of these cells(10), although the present study demonstrates a highly significant increase in biochemically determined phosphatase activity under identical conditions of treatment. There are several possible explanations for this discrepancy: 1. The histochemical procedures (fixation, washing, embedding, etc.) result in large losses of enzyme from the R-treated group because the pubic symphyses are more permeable; 2. The histochemical and biochemical substrates differ in optimal reactivity to various isozymes; 3. R may cause a release of phosphatase from the cells into the surrounding matrix and if released enzymes were no longer particulate perhaps could not be detected by histochemical means. A release of hydrolases from the cells would be

consistent with the morphological changes observed in the connective tissue of the symphysis pubic when R is administered(3,8). However, the second possible explanation is supported by the finding(16) of two acid phosphatases in mouse tissues which had different pH optima and reacted differently with different substrates.

It is tempting to propose a lysosomal mechanism for the "relaxation" phenomenon. Thus, estrogens acting in concert with GH might induce synthesis of lysosomal enzymes by the cells of bone and cartilage. The enzyme-laden cells might then attack their matrices, eroding bone and breaking down cartilagenous ground substance. When relaxin was given, lysosomal membranes might be ruptured, flooding the pubic joint with hydrolases which would cause the final breakdown of cartilage and transformation to ligamentous connective tissue. In favor of these speculations is the apparent reduction in R-induced acid phosphatase activity when pubic ligament formation is inhibted by progesterone. Goodall(17) has previously proposed that progesterone stabilizes subcellular particles to prevent post partum involution of the rabbit uterus. A strong argument against such a mechanism is the finding that progesterone itself will induce prompt involution of interpubic ligaments developed days earlier by treatment with ECP and R (unpublished observations). Such an effect of P would be difficult to explain as a consequence of increased lysosomal stability, and indeed would suggest just the reverse: that catabolic enzymes were released by P.

Summary. The pubic joint of the mouse was used as a model for the study of hormonal influences on acid phosphatase activity in connective tissue. Biochemically determined acid phosphatase was elevated in pubic joints of ovariectomized mice treated with an estrogen (ECP) and further increased by combined treatment with ECP and relaxin (R). The enhancement of enzyme activity was accompanied by transformation of the connective tissue from a cartilagenous to a ligamentous type. Progesterone (P) alone did not influence pubic joint acid phosphatase or modify the response to ECP. P appeared to reduce the expected increase in enzymatic activity in mice treated with ECP plus R and at the same time inhibited the transformation of pubic joint cartilage to ligament. Pubic joint acid phosphatase was very low in hypophysectomized mice. The enzymatic responses to ECP and ECP plus R observed in ovariectomized mice were not obtained in hypophysectomized mice unless STH was also administered. Again the changes in acid phosphatase were correlated with transformation of cartilage to ligament. The results are discussed in relation to a possible enzymatic basis for the phenomenon of pubic symphyseal relaxation.

We appreciate the skilled assistance of Margaret Butler, Sara Priester and Shirley Carter. The help and advice of Vivian Beach is gratefully acknowledged.

1. Hall, K., J. Reprod. Fertil., 1960, v1, 368.
2. ———, J. Endocrinol., 1956, v13, 384.
3. Crelin, E. S., Anat. Rec., 1963, v146, 149.
4. Horn, E. H., Endocrinology, 1958, v63, 481.
5. ———, ibid., 1960, v66, 129.
6. Horn, E. H., Barandes, M., ibid., 1961, v69, 1102.
7. Steinetz, B. G., Beach, V. L., ibid., 1963, v72, 771.
8. Steinetz, B. G., Manning, J. P., Butler, M., Beach, V. L., ibid., 1965, v76, 876.
9. Steinetz, B. G., Beach, V. L., Kroc, R. L., in Lloyd, C. H., ed., Recent Progress in the Endocrinology of Reproduction, Academic Press, New York, 1959, p389.
10. Manning, J. P., Steinetz, B. G., Butler, M., Priester, S., J. Endocrinol., 1965, v33, 501.
11. Novikoff, A. B., in Mellors, R. C., ed., Analytical Cytology, McGraw-Hill, New York, 1954.
12. ———, in Brachet, J., Mirsky, A. E., ed., The Cell, Academic Press, New York, 1961, v2, 423.
13. Manning, J. P., Babson, A. L., Butler, M., Priester, S., Can. J. Biochem. in press, 1966.
14. Steinetz, B. G., Beach, V. L., Kroc, R. L., Stasilli, N. R., Nussbaum, R., Nemith, P., Dun, R., Endocrinology, 1960, v67, 102.
15. Frantz, A. G., Rabkin, M. T., J. Clin. Endocrinol., 1965, v25, 1470.
16. Maggi, V., Franks, L. M., Carbonelle, A. W., Histochemie, 1966, v6, 305.
17. Goodall, F. R., Science, 1966, v152, 356.

Received August 25, 1966. P.S.E.B.M., 1967, v124.