Effects of Induced States on Tissue Lysozyme Activity.*†‡ (23079)

GERALD LITWACK
Department of Agricultural Biochemistry, Rutgers, State University, New Brunswick, N. J.

To establish a model system for studies of protein biosynthesis as it may be affected by hormonal conditions, experiments to test possible alteration in activity of tissue lysozymes in various hormonal states were undertaken. Although it has been observed that ACTH therapy produced a marked fall in fecal lysozyme coinciding with clinical improvement of ulcerative colitis(1) and that intravenous administration of insulin also caused a decrease in total output of the enzyme in gastric juice(2), no reports have been seen to indicate the possible role of hormones in controlling *in vivo* levels of tissue anti-bacterial enzymes.

Methods. The experimental animals, obtained from the Charles River Breeding Laboratories and of the Sprague-Dawley strain were males, weighing between 50-75 g. Hypophysectomy was carried out at 50 days of age and the animals were received at 52 days of age at which time each rat was injected with 5000 units of penicillin G (Squibb). Hypophysectomy was confirmed by adrenal weights at term and examination of the *sella turcica*. Animals were housed individually in screen-bottom cages and received diet and water *ad libitum*. Diet consisted of the following: lactalbumin (Labco, Borden Co.) 30%; vitamin mix(3) 2%; sucrose, 58%; Salts IV(4), 4%; corn oil, 5%; and fat soluble vitamins, 1% (Nopco Chemical Co. A and D concentrate and cod liver oil; 300 I.U. vit. D/g, 2250 U.S.P. units vit. A/g). Food consumption was determined on alternate days by weight loss in food cups where little or no spillage occurred. Beef GH (Armour), lot M-108 (activity = 75% of Armour standard + 0.15 U.S.P. unit/g of thyrotropic hormone contaminant) was administered as follows: 0.2 mg dissolved in 0.1 ml 0.85% NaCl was injected daily by intraperitoneal route. ACTH (Upjohn) was dissolved in physiological saline and 1 U.S.P. unit in 0.1 ml solution was injected intraperitoneally each day. Thyroid concentrate (E. Lilly; lot Z-3096; 0.17-0.23% iodine) was administered orally as 1% of diet. Adrenal weights were taken after the fresh glands were teased free of surrounding tissue and blotted. The following tissues were excised, individually wrapped in foil and placed in freezer: liver, lung, spleen, kidney and intestine (from pyloric valve to the cecum). Previous washing of the intestinal lumen caused a loss of about 5% of total enzyme activity of this portion, therefore no washing was attempted. The enzyme activity in 30 cm segments of the tract, caudad from the pyloric valve, demonstrated a different pattern in the growing rat compared to the adult rat; therefore the entire segment from pylorus to cecum was homogenized for the enzyme assay. Lysozyme activity was determined by previously described photometric method(5) using *Micrococcus lysodeikticus* as substrate prepared according to Litwack and Pramer(6). Tissue nitrogen was determined with the Folin-Ciocalteu reagent essentially as described by Lowry, *et al.* (7) and Oyama and Eagle(8). A nitrogen value is obtained by converting the Folin color which has been standardized with a tissue preparation whose Kjeldahl nitrogen value had also been determined. Linearity between the Folin color and Kjeldahl nitrogen content was obtained. Optical density values were read at 660 mμ to 0.4. Lysozyme specific activity is defined as μg equivalent of crystalline

* This investigation was supported in part by research grant from Nat. Inst. of Allergy and Infectious Diseases, P.H.S. Paper of Journal Series of N. J. Agri. Exp. Station, Rutgers Univ., State Univ. of N. J., New Brunswick.

† The following abbreviations will be used: GH (growth hormone); ACTH (adrenocorticotropic hormone); HYPOX (Hypophysectomy or hypophysectomized).

‡ The author is indebted to Dr. S. L. Steelman of Armour and Co. for a gift of beef GH. The technical assistance of Miss Sarah W. Perry in some of the enzyme assays is gratefully acknowledged.

TABLE I. Effects of Induced States on Tissue Lysozyme Activity.*

Group	No. animals	Lysozyme specific activity (μg EEWL†/mg N) Liver	Lung	Intestine	Kidney	Spleen
Normal control	11	5.3 ± 1.7‡	98 ± 9	14.8 ± 3	87 ± 15	37.2 ± 4
HYPOX	11	12.0 ± 5.3	122 ± 13	15.2 ± 9	166 ± 17	34.6 ± 5
" + ACTH	12	5.3 ± 1.7	108 ± 5	15.2 ± 4	141 ± 17	39.8 ± 3
" + GH	9	9.3 ± 1.7	96 ± 7	15.2 ± 4	119 ± 5	38.6 ± 3

* Animals on exp. 25 to 28 days. † EEWL = equivalent to crystalline egg white lysozyme.

‡ Stand. error of mean $= \frac{\sqrt{\Sigma d^2/n-1}}{\sqrt{n}}$.

egg white lysozyme (Armour) taken from a standard curve(5)/mg tissue Folin nitrogen (μg EEWL/mg N). At the time of analysis, the tissues were thawed§ for 30 min. at 5°C, teased free of surrounding tissues, blotted and weighed. Homogenates were made by grinding in 5 volumes of cold distilled water added to the tissue. These were diluted 10 times for enzyme and nitrogen determinations at 25°C. Each extract was assayed with an accuracy of ± 0.2% transmission/30 sec.(5). This accuracy was always duplicated within 2 to 3 determinations. All enzyme activities were made using the same sample of dried *M. lysodeikticus* cells. (Δ% transmission/30 sec. of 1.6 is equivalent to 5 μg of Armour egg white lysozyme.) Experiments were carried out in 2 durations, the first for 25 to 28 days and the second from 8 to 12 days.

Results. Growth data under various hormonal conditions were in agreement with those reported by Chow and Greep(9) who used a similar diet. Table I shows the results of a long-term experiment in which tissue lysozyme activity is reported as specific activity (SA). Measurements of SA show a remarkable consistency in the spleen regardless of the hormonal condition of the animal. Average body weights of animals at completion of the experiment were as follows: Normal, 248 ± 5 g (standard error of mean); HYPOX, 72 ± 4 g; HYPOX + ACTH, 80 ± 3 g and HYPOX + GH, 130 ± 4 g. The liver, lung and intestine also remain constant in the various groups. The most striking changes of the tissues tested occur in the kidney where the enzyme is most heavily concentrated(5). Upon HYPOX the SA of the enzyme is doubled even though the animals' body weight has failed to increase by about 75%. ACTH does not significantly alter this change, but GH has a slight depressing effect upon the enzyme SA. In the case of the kidney enzyme, a marked relationship of control through "capping" the enzyme activity via pituitary secretion appears to exist. The depressing effect of GH may be significant but since depression is not accomplished to the level of the untreated control, it is likely that other factors play a role in controlling the kidney enzyme SA.

A second short term experiment is shown in Table II. A study of the effects of thyroid hormone was necessary to establish if the thyrotropic hormone contaminant of GH was playing a part in the observed depression of enzyme SA. This experiment confirms the marked rise in kidney enzyme SA after 8 days of HYPOX. Levels of SA of lysozyme in liver, lung, intestine and spleen were constant in untreated and HYPOX animals. The effects of the thyroid hormone are great, especially upon the liver, lung, intestinal and kidney enzymes. When the unoperated animals received thyroid for 8 days, SA of the enzyme of all tissues except the spleen were reduced up to 50%. When the HYPOX group was treated with thyroid, a similar reduction in SA followed. The superimposed thyroid stress upon HYPOX reduced SA of the kidney enzyme to a value slightly below that observed in untreated control. When the changes in body weight are considered these differences appear to be greater. Average body weight of each group at termination of the experiment was: Normal, 157 g; HY-

§ No loss in activity was experienced before and after freezing regardless of storage time.

TABLE II. Effects of Hypophysectomy and Thyroid Hormone on Tissue Lysozyme Activity.*

Group	No. animals	Lysozyme specific activity (μg EEWL†/mg N) Liver	Lung	Intestine	Kidney	Spleen
Normal control	4	5.3 (1–12)‡	141 (81–190)	32 (11–66)	108 (92–131)	47 (39–53)
Idem + 1% thyroid	3	2.7 (2.6–4)	98 (85–105)	16 (11–21)	59 (48– 66)	40 (36–45)
HYPOX	5	9.3 (4–18)	137 (74–192)	20 (5–36)	155 (108–190)	47 (25–68)
" + 1% thyroid	7	3.8 (1–11)	109 (91–126)	6.7(3–29)	87 (65–120)	43 (24–61)

* Animals on exp. 8-12 days. † EEWL = equivalent to crystalline egg white lysozyme. ‡ Range.

POX, 82 g; HYPOX + thyroid, 67 g; and normal + thyroid, 118 g. Decreased weights due to thyroid stress were accompanied by decreased food intake. It appears, however, that this factor was not important in reduction of tissue enzyme SA because the liver xanthine oxidase activity(10,11) of these animals was tested and was not reduced when thyroid was given to normal animals.

Discussion. Increased SA of kidney lysozyme after hypophysectomy suggests that the anterior pituitary may control the SA of the kidney enzyme. It seems likely that thyrotropic hormone would play a large indirect role. The further slight suppressive effect of GH suggests that GH may play a part in this suppression. ACTH does not alter lysozyme activity in uninfected tissues, as found in ulcerative colitis(1). The "capping" effect of an acidic short chain peptide which could enter a kidney cell and inactivate the cytoplasmic lysozyme is an attractive hypothesis to explain the hypophysectomized effect. Several questions are raised as a result of these experiments, primarily, role of the kidney as a depository for the enzyme. Since the kidney appears to accumulate the greatest tissue activity of the enzyme, it may be that the kidney promotes a highly active synthesis of lysozyme. This possibility is being investigated. It is known, however, that lysozyme also circulates in blood(5,12). It is interesting to reflect that the data reported here demonstrate a remarkable consistency of the spleen SA. It might be suggested that the enzyme could be formed by the spleen and circulated to tissues which accumulate it, analogous, perhaps, to a hormone secretion where a concentration gradient is maintained between the blood stream and the concentrating tissues.

The effect of inhibition by thyroid hormone of kidney lysozyme SA and SA of other tissues occurs in untreated or HYPOX animals and is very pronounced. Fraenkel-Conrat (13) has demonstrated that lysozyme binds iodine which results in reversible inactivation. It was concluded that the imidazole group participated in the reaction. It seems possible that the thyroid hormone might bind lysozyme although why the kidney would appear as the main target tissue remains a problem. It has been shown that the action of some factors, such as insulin, in decreasing the titer of lysozyme, do so by binding the enzyme and in some cases precipitating it (14). If it may be assumed that kidney lysozyme is also in the bound form, the measurable activity of the tissue is due to the free form of the enzyme which becomes dissociated because of effects of various factors, in this case hormonal effects, upon association of the enzyme. One could then view hypophysectomy as having a "dissociating" effect and thyroid hormone as having a depressing or "associating" effect. In this regard it is of interest that Smith and Dubos(15) have reported that thyroxine treatment "interfered with the bactericidal mechanism in the liver, spleen and kidneys of mice during the initial phase of infection." In addition, they found that thyroid extract or dinitrophenol were inactive in causing the infecting organisms (staphylococci) to multiply more rapidly in the various organs.

Summary. 1. In growing rats, hypophysectomy has been shown to significantly increase the level of kidney lysozyme specific activity(SA) up to 2-fold. Other tissues, particularly the spleen, show constant SA regardless of hormonal condition of the animal. 2. Thyroid has a marked depressing

effect upon kidney lysozyme SA when given to either untreated or hypophysectomized growing animals.

1. Gray, S. J., Reifenstein, R. W., Benson, J. A., Jr., and Young, J. C., *Am. Med. Assn. Arch. Intern. Med.*, 1951, v87, 646.

2. Gray, S. J., Reifenstein, R. W., Gordon, J. C., Spiro, H. M., and Connolly, E. P., *J. Clin. Invest.*, 1950, v29, 1595.

3. Williams, J. N., and Elvehjem, C. A., *J. Biol. Chem.*, 1949, v181, 559.

4. Phillips, P. H., and Hart, E. B., *ibid.*, 1935, v109, 657.

5. Litwack, G., PROC. SOC. EXP. BIOL. AND MED., 1955, v89, 401.

6. Litwack, G., and Pramer, D., *ibid.*, 1956, v91, 290.

7. Lowry, O H., Rosebrough, N. J., Farr, A. L., and Randall, R. J., *J. Biol. Chem.*, 1951, v193, 265.

8. Oyama, V. I., and Eagle, H., PROC. SOC. EXP. BIOL. AND MED., 1956, v91, 305.

9. Chow, B. F., and Greep, R. O., *ibid.*, 1948, v69, 191.

10. Litwack, G., Fatterpaker, P., Williams, J. N., Jr., and Elvehjem, C. A., *J. Nutri.*, 1954, v52, 187.

11. Remy, C., Richert, D. A., Westerfeld, W. W., and Tepperman, J., *Endocrinology*, 1950, v73, 673.

12. Flanagan, P., and Lionetti, F., *J. Hematol.*, 1955, v10, 497.

13. Fraenkel-Conrat, H., *Arch. Biochem.*, 1950, v27, 109.

14. Ross, V., PROC. SOC. EXP. BIOL. AND MED., 1949, v72, 465.

15. Smith, J. M., and Dubos, R. J., *J. Exp. Med.*, 1956, v103, 119.

Received February 4, 1957. P.S.E.B.M., 1957, v94.

Development and Serial Cell-Free Passage of a Highly Potent Strain of Mouse Leukemia Virus.* (23080)

LUDWIK GROSS

Cancer Research Unit, Veterans Administration Hospital, Bronx, New York City.

After it was found that filtered extracts prepared from tissues of leukemic Ak or C58 mice may induce leukemia following inoculation into newborn mice of a susceptible strain (1-4), it became apparent that the results of individual experiments may vary to a considerable degree, depending on the particular extract used. Of 70 filtrates tested, each prepared from a different donor, 18 were found completely inactive(5). Similar observations were made by Woolley(6). Furthermore, extracts prepared from C3H mice in which leukemia had been induced with cell-free Ak leukemic agent, proved more active than those prepared from Ak mice with spontaneous leukemia(4-5). It appeared reasonable to assume, therefore, that selecting a particularly active extract and passing the agent through several successive newborn hosts, may eventually result in a strain of leukemic virus of high potency(5,7). To find a potent extract, groups of litters of newborn C3H mice were inoculated with cell-free leukemic extracts prepared from different donors. Among inoculated mice, those developing leukemia at earliest date were then used as donors for preparation of new cell-free extracts, which were inoculated into newborn mice. The first mice developing leukemia were used as donors for preparation of new cell-free extracts, which were inoculated into newborn mice, etc. A number of different passage-strains of leukemic agents were thus obtained. Of the leukemic passage-strains developed during these experiments, the one here reported and designated "passage A" proved to be highly pathogenic. Filtered extracts prepared from the last few passages of this agent induced acute, generalized, lymphatic leukemia in most of the inoculated mice after only 3½ months. Furthermore, extracts containing the passage virus could be preserved by lyophilization, or simply by freezing in carbon dioxide ice at −70°C in sealed ampoules without apparent loss of activity.

* Aided, in part, by grants from Damon Runyon Memorial Fund, and Am. Cancer Soc.

Methods. Preparation of leukemic extracts.