The observation that these adrenocortical carcinomas which have been known to secrete sex hormones, also contain appreciable amounts of glucocorticoids, is of interest in view of the findings of Dickie and Woolley on the pituitaries of mice bearing adrenocortical cancers(8). The changes which they found in the pituitary basophils in these mice resembling those in human cases of Cushing's syndrome, may perhaps be correlated with glucocorticoid secretion by the cortical tumors rather than with the sex hormone secretion. In man, changes in the pituitary basophils, especially the hyaline changes described by Crooke, are characteristic of Cushing's syndrome and have also been found in the pituitaries of individuals treated with large doses of cortisone(9,10). It is of interest too that an elevated level of formaldehydogenic steroids in the urine has been observed in some instances of adrenogenital syndrome due to adrenocortical carcinoma in women, although the clinical picture gave no evidence of excessive glucocorticoid effects. One may draw a tentative parallel to the mouse adrenocortical cancers reported in this paper.

Summary. Adrenocortical carcinomas, induced in mice by early castration, and transplanted to recipient mice, were assayed for glucocorticoids by the mouse liver glycogen method of Venning. Glucocorticoids were present in all tumors. The hormone content of the tumors was compared with that of normal mouse adrenals and was found to be smaller on the basis of tissue weight. Incubation of tumor homogenate with desoxycorticosterone glucoside failed to yield increased glucogenic activity.

1. Fekete, E., Wooley, G., and Little, C. C., *J. Exp. Med.*, 1941, v74, 1.

2. Woolley, G. W., and Little, C. C., *Cancer Res.*, 1945, v5, 193.

3. Spiegel, A., *Arch. f. pathol. Anatomie*, 1939, v305, 367.

4. Gardner, W. U., *Cancer Res.*, 1941, v1, 632.

5. Frantz, M. J., and Kirschbaum, A., PROC. SOC. EXP. BIOL. AND MED., 1948, v74, 357.

6. ———, *Cancer Res.*, 1949, v9, 257.

7. Dorfman, R. I., and Gardner, W. U., *Endocrinology*, 1944, v34, 421.

8. Dickie, M. M., and Woolley, G. W., *Cancer Research*, 1949, v9, 372.

9. Golden, A., Bondy, P. K., and Sheldon, W. H., PROC. SOC. EXP. BIOL. AND MED., 1950, v74, 455.

10. Laqueur, G. L., *Science*, 1950, v112, 429.

11. Huseby, R. A., and Bittner, J. J., *Cancer Res.*, 1950, v10, 226.

12. Venning, E. H., Kazmin, V. E., and Bell, J. C., *Endocrinology*, 1946, v38, 791.

13. Furth, J., Gadsen, E. L., and Upton, A. C, PROC. SOC. EXP. BIOL. AND MED., 1953, v84, 253.

14. Hayano, M., Dorfman, R. I., and Yamada, E. Y., *J.B.C.*, 1951, v193, 175.

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Development of Experimental Exophthalmos in Scorbutic Guinea Pigs.* (20904)

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Since the publication of Wolbach's classic investigation of scurvy(1,2), it has been accepted that in the scorbutic state there is a deficiency in the formation of mesenchymal intercellular substances. Production of both major elements of the connective tissue intercellular material, the fibrillar components as well as the amorphous ground substance, has been reported to be diminished or absent. There has been ample confirmation of the fact that collagen fibre formation in scurvy

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is markedly reduced or ceases altogether. However, it is not yet so clearly evident whether a similar interference with the production of the interfibrillar amorphous ground substance also takes place. Penney and Balfour(3) found no evidence of mucopolysaccharide formation in the wounds of totally scorbutic guinea pigs. Bunting(4), on the other hand, reported greater than normal quantities of metachromatic material in wounds of partially scorbutic animals. In similar studies in this laboratory, the results were difficult to interpret because of the complexity and multiplicity of the processes involved in the healing of wounds, so that a simpler approach was sought.

It has been reported(5) that the production of experimental exophthalmos in guinea pigs is accompanied by the appearance of increased quantities of mucinous ground substance in the orbital connective tissue. This deposition of ground substance takes place in connective tissue already present in the orbit, so that the problems arising from the cellular and vascular proliferation and the formation of new collagen which complicate studies of wound healing are not involved. The study of experimental exophthalmos was therefore felt to provide a less complicated approach to the problem of ground substance formation in the scorbutic animal than did earlier methods. Histologic and chemical studies of the orbital tissues of exophthalmic, scorbutic guinea pigs were carried out.

Methods. Forty guinea pigs (250 g) of both sexes were divided into four groups: intact animals on a diet of purina chow and fresh greens, excluding cabbage; thyroidectomized animals on the same diet and treated with 3 mg/day of a preparation of the thyrotropic fraction of the adenohypophysis (TSH);† intact guinea pigs on a scorbutic diet supplemented by cod liver oil and vitamin B complex; and thyroidectomized animals treated with TSH and a similar scorbutic diet. Half of the animals were thyroidectomized and were treated with parathyroid extract and calcium for 4 days postoperatively. Nine days were allowed to elapse for wound healing. Half of the thyroidectomized guinea pigs and half of the unoperated animals were then started on the scorbutogenic diet; at the end of 18 days on this regimen these animals had lost an average of 69 g each. At this time all thyroidectomized guinea pigs were injected with 3 mg/day of TSH intraperitoneally for 5 days. The animals were lightly etherized and exsanguinated. The orbital contents were carefully dissected out *en bloc*, and the eyeball freed of the ocular muscle insertions and discarded. The lacrimal and Harderian glands were separated so that the tissues were divided into three portions: lacrimal gland, Harderian gland, and an ocular muscle-connective tissue fraction. The contents of one orbit were utilized for analyses of water and hexosamine content, and those of the other for histologic study. Total water contents were determined by weighing on an analytic balance before and after desiccation in a drying oven at 100-105°C for 12 hours followed by a similar period in a vacuum desiccator. The hexosamine contents were determined by a modification of the method of Elsom and Morgan. Tissues were fixed in 10% formalin, cold absolute alcohol, and Zenker-acetic acid, and were embedded in paraffin. Bones were decalcified in formic acid. Sections were cut at 5-7 μ. The strains used included hematoxylin-eosin, toluidine blue, periodic acid-Schiff reagent, and the Gomori technic for phosphatase. Frozen sections of the formalin-fixed tissues were stained for fat with Sudan IV.

Results. Grossly visible exophthalmos became apparent in all thyroidectomized animals receiving TSH after 3 days of treatment. There were no obvious differences in the degree of exophthalmos between the non-scorbutic and scorbutic groups. The eyes of the normal controls and the untreated scorbutic guinea pigs exhibited no such changes. During dissection it was apparent that the retrobulbar tissues of the exophthalmic animals were more mucoid than those of the controls.

Histologic examination of the orbital tissues revealed findings identical with those previously described(5). The changes observed were most prominent in the loose connective tissue of the muscle-connective tissue fraction, al-

† Thyrotropic hormone obtained through the courtesy of Dr. L. W. Donaldson of Parke, Davis Co.

though a qualitatively similar response was seen in the connective tissue of the septa of the glandular fractions. The glandular tissue itself did not participate in the reaction.

Fibroblasts in the tissues of all scorbutic animals revealed marked vacuolization, without any relationship to the presence or absence of exophthalmos. The vacuoles were demonstrated to contain neutral fat upon staining with Sudan IV.

There were prominent subconjunctival lymphatic follicles in both the normally fed and scorbutic animals. In the two groups which had undergone thyroidectomy and been treated with TSH, these follicles were considerably increased in size. The diffuse lymphatic cell infiltration reported by others was not observed. In some of the animals, however, particularly those of the exophthalmic groups, there was a slight infiltration with polymorphonuclear leukocytes, most prominently in the loose subconjunctival tissue. This evidence of conjunctivitis was more frequent in the scorbutic animals, possibly because of their lowered resistance to infection.

Sections from control animals stained with toluidine blue revealed at most very faint traces of metachromatic material in the extracellular portions of the connective tissue. The tissues from the intact scorbutic group also displayed minimal metachromasia. In the thyroidectomized group treated with TSH and fed a normal diet, large deposits of metachromatic material were noted. This was most prominent in the loose connective tissue, adjacent to the muscle, and in the septa of the adipose tissue. The TSH treated thyroidectomized scorbutic animals also revealed considerable amounts of metachromatic substance in the same regions. In fact, the latter two groups were indistinguishable histologically, except for the vacuolization of the fibroblasts of the scorbutic group.

The retrobulbar tissues stained by means of the periodic acid-Schiff reagent reaction revealed moderate amounts of PAS-positive material in the intact animals, whether normal or scorbutic. In the thyroidectomized, TSH-treated groups there was a striking increase of the quantities of the material in the extracellular spaces of the connective tissue. This was more evident in alcohol-fixed than formalin-fixed tissues, indicating that some of this material is water soluble. PAS-positive granules were observed in the cytoplasm of the fibroblasts of both normal and scorbutic animals. A significant difference in the number of such granules in the two groups was not apparent.

The phosphatase preparations were inconclusive. It appeared that there was an increase in the amounts of alkaline phosphatase in the cytoplasm of the fibroblasts of the animals with exophthalmos, but these results were neither striking nor consistent. We were able to demonstrate no correlation between the existence or absence of scurvy and the degree of phosphatase activity in the sections.

The results of tissue analyses for water and hexosamine contents were consistent with earlier observations (Table I). There were significant increases in the total water content and total hexosamine concentration in the orbital tissues of all exophthalmic guinea pigs, both normally fed and scorbutic. In these groups the rise in water and hexosamine contents of the tissues from scorbutic animals was of the same magnitude as that seen in the normally fed group. The water content of the connective tissue-muscle fraction of the retrobulbar tissues of the untreated scorbutic animals was slightly lower than the normal control values, possibly reflecting a generalized dehydration ensuing from the poor physical state of the former group. The hexosamine concentrations per unit of dry weight were identical for these two groups.

Discussion. The results of these investigations indicate that the existence of the scorbutic state *per se* does not interfere with the deposition of intercellular ground substance in connective tissue. The development of exophthalmos in scorbutic guinea pigs implies that in scurvy there is no impairment of the capacity of the retrobulbar connective tissue to respond to the exophthalmogenic factor present in the thyrotropic fraction of the adenohypophysis.

Scorbutic TSH-treated guinea pigs exhibited histologic and chemical responses identical to those reported earlier in normally fed animals. Metachromatic material observed in the

TABLE I. Water and Hexosamine Contents of Muscle-Connective Tissue Fraction of the Orbital Contents.

		No.	Wet wt, mg	Dry wt, mg	Water, %	Hexosamine, mg/100 mg
Control	Intact	8	157	33.1	79.0 ± .6*	.64 ± .02*
	Thyroid × TSH	7	230.9	32.7	86.0 ± 1.0	1.11 ± .08
Scurvy	Intact	6	147.9	35.4	75.6 ± 1.7	.65 ± .03
	Thyroid × TSH	6	203.4	32.0	84.4 ± .9	1.15 ± .05

* Stand. error of the mean.

tissues of scorbutic animals did not appear to differ either quantitatively or qualitatively from that seen in non-scorbutic exophthalmic animals. Sections stained by the periodic acid-Schiff reagent technic demonstrated striking increases in the amounts of PAS-positive material in the tissues of exophthalmic animals. This, too, appeared to be as great in the scorbutic animals as in the normally fed controls.

It is significant that the localization of the sites of deposition of the metachromatic material did not coincide with that of the PAS-positive substances. Deposits of fuchsinophilic material were often seen surrounding a clear zone which was filled with metachromatically staining matter in a corresponding section stained with toluidine blue. These findings imply that the two staining methods indicate the presence of different substances, or of the same substance in different states of polymerization, as has been pointed out by Gersh and Catchpole(6). It is generally thought that toluidine blue reacts metachromatically with highly polymerized polysaccharides probably in the form of protein complexes (mucoproteins). On the other hand the PAS method stains reactive groups within less highly polymerized substances which, as Gersh and Catchpole have indicated, are probably glycoproteins. However, this method often fails to stain tissues known to contain mucoproteins. For example, the intermediate zone of the cock's comb, which is extremely rich in polysaccharide, remains PAS negative in formalin-fixed, alcohol-fixed, and freeze-dried preparations.

The histologic findings are further substantiated by the results of the water and hexosamine determinations. The increase in the concentrations of both is of the same magnitude in the scorbutic and normally fed groups, again indicating that there is no apparent deficiency in the capacity of the scorbutic animal to produce polysaccharide-containing intercellular substance.

Summary. 1. Scorbutic thyroidectomized guinea pigs treated with TSH developed exophthalmos as readily as a group of similarly treated animals maintained on an adequate diet. 2. Histologic and histochemical findings in orbital tissues of the scorbutic guinea pigs were identical with those seen in the control group. 3. The increase in water and hexosamine contents of orbital tissues of exophthalmic animals was as great in those with scurvy as in the group fed an adequate diet. 4. The existence of the scorbutic state does not in itself affect the capacity of the connective tissue to produce the substances which constitute the amorphous interfibrillar ground substance.

1. Wolbach, S. B., and Howe, P. R., *Arch. Path.*, 1926, v1, 1.

2. Wolbach, S. B., *Am. J. Path.*, 1933, Suppl. 9, 689.

3. Penney, J. R., and Balfour, B. M., *J. Path. and Bact.*, 1949, v61, 171.

4. Bunting, H., and White, R. F., *Arch. Path.*, 1950, v49, 590.

5. Ludwig, A. W., Boas, N. F., and Soffer, L. J., PROC. SOC. EXP. BIOL. AND MED., 1950, v73, 137.

6. Gersh, I., and Catchpole, H. R., *Am. J. Anat.*, 1949, v85, 457.

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