the patterns obtained with proteins(12). This suggests the possibility that the product of the chromatopile has a higher molecular weight than the peptides so far studied in the solvent systems reported in this paper. This possibility is strengthened by the observation that the ninhydrin color produced by Fraction 3 is about one-tenth that obtained from an equal weight of an amino acid. A quantitative determination of ninhydrin coloration by the method of Moore and Stein(13) showed that 1 mg of Fraction 3 gives a color equal to that produced by about 0.06 mg alanine. If a mole-for-mole equivalence of color can be assumed, this would indicate a molecular weight of about 1500 for Fraction 3. Similar determinations on the starting materials for the chromatopile give a value of about 1200. Attempts are being made to devise new systems capable of resolving Fraction 3. Meanwhile, such samples provide excellent starting materials for studying the effects of enzymatic and acid hydrolysis.

Summary. Pepsin-treated ACTH materials at approximately 10 times standard were fractionated in a chromatopile using the system butanol-acetic acid-water. The activity was found to be associated with a slow-moving ninhydrin-positive area from which fractions having potencies of about 50 times standard were recovered. No further chromatographic resolution of these fractions was obtained in the solvent systems ordinarily used for amino acids and peptides. In all cases, the activity was associated with ninhydrin-positive areas of the chromatograms.

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12. Franklin, A. E., and Quastel, J. H., PROC. SOC. EXP. BIOL. AND MED., 1950, v74, 803.

13. Moore, S. and Stein, W., *J. Biol. Chem.*, 1948, v176, 367.

Relationship of Ascorbic Acid to Secretion of Adrenocortical Hormones in Guinea Pigs.* (19160)

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Ascorbic acid was first isolated from animal tissues by Szent-Gyorgi who found the vitamin in significant quantities in the adrenal gland (1). Subsequently it was shown that ascorbic acid is present in greater concentration in the adrenal cortex than in any other tissue(2). It had been observed also that the adrenals hypertrophy in scurvy. This change has come to be recognized as one of the pathologic criteria of vit. C deficiency(3-5). These facts have stimulated interest in the relationship of ascorbic acid to adrenocortical function with the result that numerous investigations have dealt with the problem(6-13).

This paper reports the results of a study in which the secretion of adrenocortical hormones was determined in guinea pigs on

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1. Szent-Gyorgyi, A. J., *J. Biol. Chem.*, 1928, v22, 1387.

2. Follis, R. H., *The Pathology of Nutritional Disease*, Chas. C. Thomas, Springfield, Ill., 1948.

3. Quick, A. J., PROC. SOC. EXP. BIOL. AND MED., 1933, v30, 373.

4. Bessessen, D. H., *Am. J. Physiol.*, 1923, v63, 245.

5. Teppermen, J., Engel, F. L., and Long, C. N. H., *Endocrinology*, 1946, v38, 1.

6. Giroud, A. and Ratsimamanga, A. R., *Presse Med.*, 1940, v48, 449.

7. Giroud, A., and Santa, N., *Compt. Rend. Soc. de Biol.*, 1939, v131, 1176.

8. Giroud, A., Santa, N., Martinet, M., and Bellon, M. T., *Compt. Rend. Soc. de Biol.*, 1940, v134, 100.

9. Lowenstein, B. E., and Zwemer, R. L., *Endocrinology* (abstract), 1946, v39, 63.

10. Long, C. N. H., *Fed. Proc.*, 1947, v6, 461.

11. Jailer, J. W. and Boas, N. F., *Endocrinology*, 1950, v46, 314.

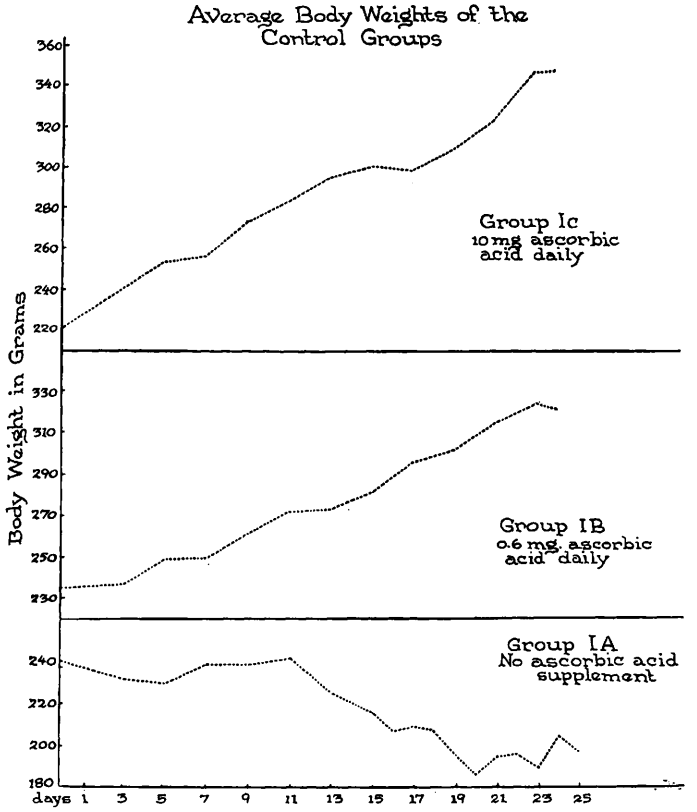

FIG. 1.

graded levels of intake of ascorbic acid. The observations indicate that deficiency of this vitamin functions as do other types of stress but that the vitamin is not directly involved in the elaboration of hormones of this gland.

Procedure. Young male guinea pigs weighing from 200-285 g were used in these experiments. All animals were fed *ad libitum* a scorbutigenic diet described by Zilva(14). One group of 20 guinea pigs was given ACTH while another group of 23 served as controls. Animals in each group were placed in one or another of 3 subgroups which were provided graded supplements of ascorbic acid. The first subgroup (A) received no supplements; each animal in the second (B) was given 0.6 mg of ascorbic acid daily; and those in the third (C) received a daily supplement of 10 mg. These doses were selected so as to provide approximately the minimum daily requirement for the guinea pigs of subgroup B and a more than adequate intake for the animals in subgroup C. The solutions of ascorbic acid were injected intraperitoneally and were prepared immediately before injection by dilution in isotonic saline. Administration of ACTH in the treated group began on the fifteenth day of the scorbutigenic diet. By this time all animals in the group without supplementary ascorbic acid had attained maximum weight and in most instances were beginning to lose weight. The hormone was administered intramuscularly or subcutaneously in quantities of 2 mg per day distributed in 4 equal doses given at intervals of approximately 6 hours. In preparation of ACTH for injection, the hormone was tritu-

12. Hyman, G. A., Ragan, C., and Turner, J. C., PROC. SOC. EXP. BIOL. AND MED., 1950, v75, 450.

13. Treager, H. S., Gabuzda, G. J., Zamcheck, N., and Davidson, C. S., PROC. SOC. EXP. BIOL. AND MED., 1950, v75, 517.

14. Penney, J. R., and Zilva, S. S., *Biochem. J.*, 1946, v40, 695.

rated in isotonic saline, the solutions adjusted to pH-3 and diluted to the desired volume. This preparation was then frozen and stored until used. Animals were weighed daily throughout the experimental period. The level of circulating eosinophiles was determined at intervals of from 2 to 4 days and served as a measure of adrenal cortical hormone secretion. Eosinophile counts were done by a modification of the method described by Randolph(15). Scorbutic animals were sacrificed by a blow on the head when moribund. When a majority of the animals with scurvy in each group had been killed, the remaining animals in that group were sacrificed. Autopsies were performed immediately after death and the adrenals and a portion of the liver were removed, weighed, and prepared for analysis. Ascorbic acid analyses were done on these tissues by the method of Roe and Keuther(16).

Results. Control group. The variations in growth of the control group during the experimental period are demonstrated in Fig. 1. Guinea pigs which received 0.6 mg of vit. C as a daily supplement (Group IB) gained an average of 97 grams while those given 10 mg of vit. C daily (Group IC) gained an average of 128 g. Animals not receiving ascorbic acid supplements (Group IA) gained weight at a slower rate for eleven days, after which progressive weight loss ensued.

The number of circulating eosinophiles decreased progressively throughout the experiment in 5 of the 8 animals on the ascorbic acid-free regimen. (Fig. 2) The remaining 3 guinea pigs were found at the initiation of the experiment to have low eosinophile counts which remained without significant change. The average eosinophile count at the onset of the experimental period was $127 \pm 11.3/mm^3$. As severe scurvy developed all counts approached zero and remained at this low level until death. The average count shortly before death was $10.6 \pm 3.2/mm^3$. Determinations on animals receiving supplements of

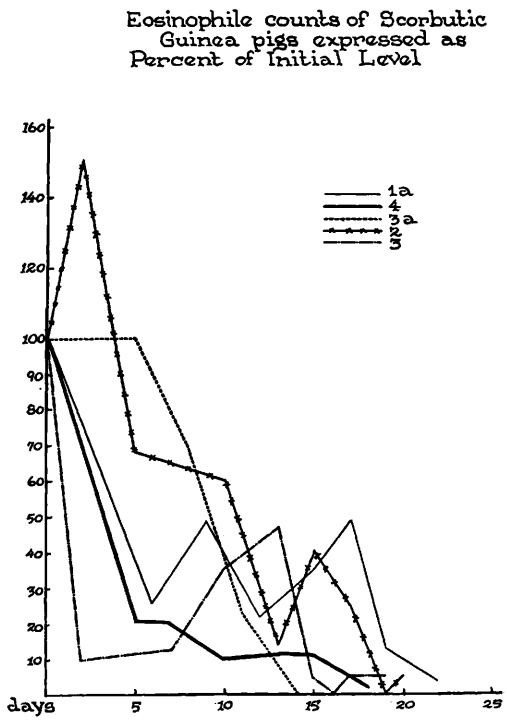

FIG. 2.

ascorbic acid were variable. Most had higher levels during the latter part of the experiment than at the start. No animal to which ascorbic acid was administered was found to have progressive decline in the eosinophile count. All animals without supplements of vit. C developed scurvy and survived for 17-25 days with a mean survival period of 20.5 days. Typical gross lesions of scurvy including hemorrhages into joints and intestines as well as adrenal hypertrophy were found. The actual adrenal weights and the adrenal weights in mg per gram of body weight in animals receiving ascorbic acid were significantly less than those of the scorbutic animals. (Shaded areas Fig. 3 and Table I). The concentration of vit. C in the adrenal cortex and liver varied directly with the daily intake of this vitamin.

ACTH-treated group. The guinea pigs in this group without supplementary ascorbic acid attained maximum weight on the fourteenth day. ACTH administration began one day later. The rapid loss of weight noted in the control animals as scurvy developed was not seen in the treated guinea pigs. Actually 4 of the 7 animals showed cessation of weight

15. Randolph, T. G., *J. Lab. and Clin. Med.*, 1949, v34, 1697.

16. Roe, J. H., and Keuther, C. A., *J. Biol. Chem.*, 1943, v147, 399.

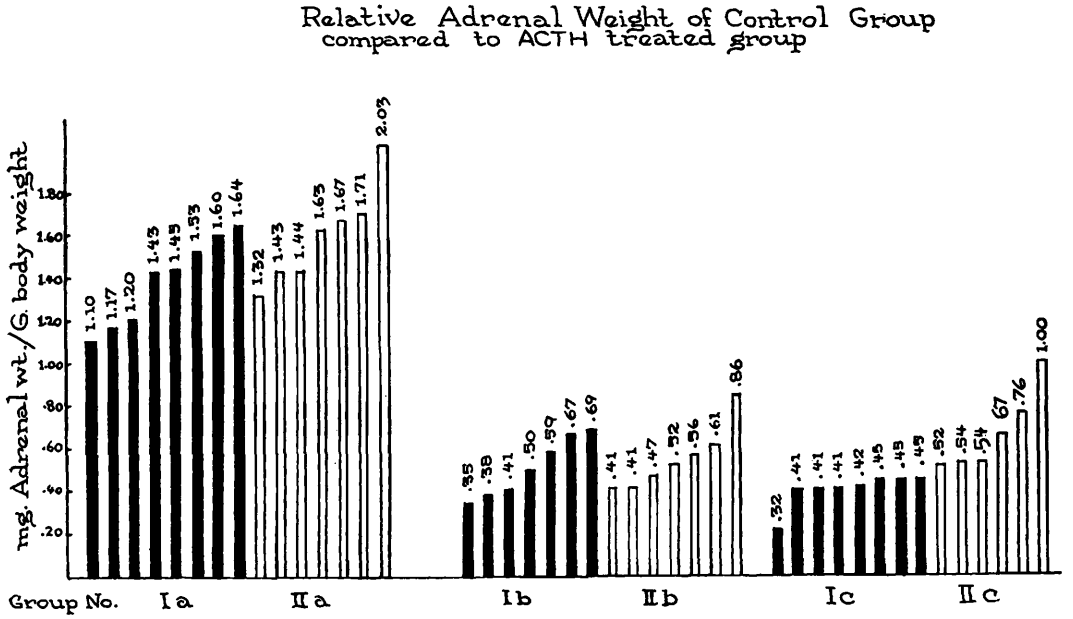

FIG. 3.

loss and for a period of 2 to 5 days again gained weight. Following this secondary peak in weight, progressive loss of body weight ensued and continued until death. (Fig. 4) Of the remaining three animals, two continued to lose weight after ACTH administration began but the weight loss proceeded slowly. The other animal showed a progressive decrease in weight which was seemingly unaffected by ACTH administration.

The number of circulating eosinophiles of this group of animals was variable prior to administration of ACTH. During this period the average number of eosinophiles was 68 $\pm$ 8.24/mm^3. However, within two or three days after institution of ACTH therapy, eosinophile counts in all animals fell to lower levels, the average value at this time being 34 $\pm$ 5.8/mm^3. Following the initial decrease there was a progressive increase in eosinophile count in guinea pigs which had not received supplements of ascorbic acid. It was considered that this rise might represent evidence of development of adrenal insufficiency. Therefore, to determine if the adrenal cortex retained its ability to respond to the hormone a large additional dose of ACTH (12.5 mg) was administered intramuscularly to 4 of the scorbutic animals. Eosinophile determinations were done prior to and 4 hours after the injection. In each animal there occurred a decrease of more than 50% in the eosinophile count.

Survival of the ACTH treated scorbutic animals was from 26-35 days with a mean survival period of 28.1 days. When these guinea pigs were sacrificed it was found that adrenal hypertrophy was somewhat greater than in the corresponding control animals. Actual and relative adrenal weights of animals on 0.6 mg ascorbic acid daily were approximately the same as those of the corresponding control group while the animals on a daily supplement of 10 mg of ascorbic acid were found to have greater actual and relative adrenal weights than the corresponding control series. (Fig. 3 and Table I).

Concentrations of ascorbic acid in adrenals and livers of these animals varied directly with the intake of vit. C (Table I). The ascorbic acid content of the tissues of guinea pigs receiving 10 mg of vit. C and ACTH were less than the corresponding control animals but this difference was not statistically significant.

Discussion. These observations indicate an inverse relationship between the daily intake of vit. C and adrenal weight. The mean adrenal weight (actual and relative) of the

TABLE I. Adrenal Weights and Concentration of Ascorbic Acid in Adrenal Glands and Liver of Guinea Pigs in Control and ACTH Treated Groups.

Group	No. of animals	Daily supplement of ascorbic acid (mg)	ACTH admin.	Mean adrenal wt ± S.E. (mg)	Mean relative adrenal wt ± S.E. (mg/g body wt)	Mean adrenal ascorbic acid content ± S.E. (mg/100 g)	Mean liver ascorbic acid content ± S.E. (mg/100 g)
I A	8	0	0	246.3 ± 11.75	1.39 ± .096	1.18 ± 44	0
IIA	7	0	+	314.3 ± 12.3	1.6 ± .12	.83	.3
I B	7	.6	0	163.6 ± 16.3	.512 ± .058	19.2 ± 1.8	3.31 ± .64
IIB	7	.6	+	172 ± 10	.55 ± .07	17.5 ± 2.58	2.29 ± .29
I C	8	10	0	144.8 ± 7.6	.409 ± .02	124.2 ± 21.8	19.3 ± 2.53
IIC	6	10	+	211.2 ± 12.8	.67 ± .099	82.8 ± 8.95	14.4 ± 2.18

control guinea pigs which received no supplements of ascorbic acid was much greater than that of control animals receiving supplements. While weights of the glands of animals on 0.6 mg of ascorbic acid daily were greater than those of the group receiving 10 mg each day, this difference was not of sufficient magnitude to be statistically significant. It is conceivable that this latter difference might have been greater had the experimental period been longer. A recent investigation had demonstrated that not until the ascorbic acid-cholesterol ratio in the adrenal cortex falls to below 1:500 does adrenal hypertrophy occur (17). An interpretation of these findings which seems wholly tenable is that a diet deficient in vit. C acts as a form of stress to which the adrenal responds by hypertrophy comparable to that which occurs in other conditions of chronic stress. Administration of ACTH produced adrenal hypertrophy in the animals at the highest level of supplementation of ascorbic acid. Guinea pigs on the completely deficient diet and those on an intake of 0.6 mg of vit. C daily responded to this hormone by somewhat greater adrenal hypertrophy than corresponding controls, but the differences were slight and not of significance. It seems possible that this small dose of ACTH is sufficient to produce adrenal hypertrophy in animals with adequate intake of ascorbic acid but when given to animals already adapting to chronic stress and in which the pituitary is already secreting moderate to large amounts of this hormone, the dose is insufficient to produce further hypertrophy. When it was first noted that ACTH caused a temporary cessation in loss of body weight, this was attributed to fluid retention. However, it soon became apparent that continued administration of this hormone delayed the onset of severe symptoms of scurvy, and moreover the mean survival period of the treated animals was 8 days greater than that of the controls. It is postulated that the temporary alleviation of scurvy resulted from release of ascorbic acid from the adrenals under influence of the hormone. It has been shown by others that ACTH reduces the adrenal cortical content of ascorbic acid(18) and Beck has reported that ACTH administration increased the urinary output of ascorbic acid in an infant with scurvy(19).

The relationship of ascorbic acid to the function of the adrenal cortex has been investigated by many authors with conflicting results. Giroud *et al.*(6-8), Dugal and Therien(20), and Lowenstein and Zwemer(9) have indicated that adrenal cortical function is dependent on an adequate supply of ascorbic acid. On the other hand, Long(10), Jailer (11), Hyman(12), and Treager(13) have accumulated evidence which indicates that this vitamin is of little or no importance in secretion of adrenal cortical hormones.

17. Stepto, R. C., Pirani, C. L., and Consolazio, C. F., *Fed. Proc.*, 1951, v10, 371.

18. Sayers, G., Sayers, M. A., Lang, T. Y., and Long, C. N. H., *Endocrinology,* 1946, v38, 1.

19. Beck, J., Browne, J., Hackney, J., and MacKenzie, K. K., *Proc. Second Clinical ACTH Conference,* Vol. 1—*Research,* The Blakiston Co., New York, 1951.

20. Dugal, L. P., and Therien, M., *Endocrinology,* 1949, v44, 420.

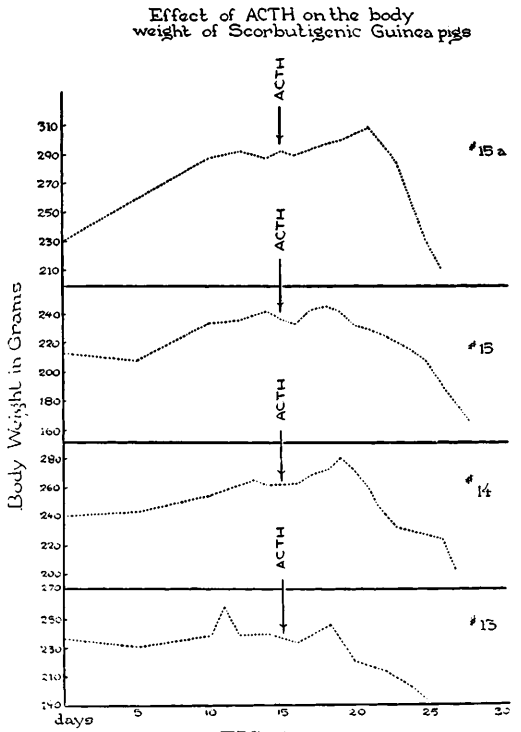

FIG. 4.

A fall in the level of circulating eosinophiles has been shown to be a sensitive measure of the secretion of adrenal hormones with an oxygen atom at the C-11 position(21). The ability of the scorbutic animals in the experiments herein reported to maintain their circulating eosinophiles at very low levels until the time of death may be due to the nutritional deficiency *per se* or may indicate continued production of cortical hormones. When it was thought adrenal insufficiency might be developing in scorbutic animals treated with ACTH, a large additional dose of the hormone was capable of stimulating production of 11-oxysteroids by the adrenal. Thus, the ability of the adrenal to respond to endogenous and exogenous ACTH with secretion of 11-oxysteroids at a time when severe scurvy exists would seem to support the view that ascorbic acid is not essential for the adrenal cortex to secrete hormones of this type.

Summary and conclusions. (1) The weight of the adrenal glands of guinea pigs on graded levels of intake of ascorbic acid varied inversely with daily intake of this vitamin. (2) A progressive decrease in the number of circulating eosinophiles occurred in guinea pigs maintained on a scorbutigenic diet. (3) There was a delay in onset of severe symptoms of scurvy and an increase of the mean survival period in scorbutic animals treated with ACTH. (4) The administration of a large dose of ACTH to guinea pigs with severe scurvy was promptly followed by a decline of more than 50% in the level of circulating eosinophiles. (5) These observations are interpreted as indicating that ascorbic acid deficiency functions as a non-specific stress and that this vitamin is not directly involved in mechanisms of elaboration of adrenal cortical hormones with an oxygen atom at the C-11 position.

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21. McDermott, W. V., Fry, E. G., Brobeck, J. R., and Long, C. N. H., *Yale J. Biol. and Med.*, 1950, v23, 52.