experiment was conducted during the regular school term it is obvious that these control data cannot represent truly an unstressed level of serum cholesterol. It does seem likely, however, that the periods of observation do coincide with periods of relatively lesser and greater degrees of mental and emotional stress.

As one might expect, these individuals vary greatly in their clinical and biochemical responses to stress. It is apparent that the situational stress of examination week must have different meaning and hence a differing stress value for the subjects. Further investigation is needed to establish whether this increase in serum cholesterol is harmful or simply part of a normal and desirable bodily adjustment to mental and emotional stress.

The difference between these results and those of Beischer(6) may be due, as he has suggested, to his use of fewer subjects and of a particularly and relatively transient stress experience. Our data would coincide with changes which Selye(5) has described in connection with the "resistance phase" of the general adaptive response to various forms of systemic stress.

Summary. A significant increase in mean value for serum cholesterol is shown to accompany the mental and emotional stress of examination week in a group of 44 apparently healthy, male, medical students. The mean value for serum cholesterol increased to 238 mg %, which represents an 11% increase over the mean control value of 214 mg % ($P < 0.001$).

1. Keys, A., *Minnesota Med.,* 1955, v38, 758.
2. Katz, L. N., Stamler, J., Pick, R., *J.A.M.A.,* 1956, v161, 25.
3. Selye, H., *The Physiology and Pathology of Exposure to Stress,* Acta Inc., Medical Publishers, Montreal, Canada, 1950.
4. Kuhl, Jr., W. J., Beck, E. M., Gershberg, H., Street, E., Ralli, E. P., *Metabolism,* 1955, v4, 143.
5. Selye, H., *ibid.,* 3, p278.
6. Beischer, D. E., *J. Av. Med.,* 1955, v27, 260.
7. Pearson, S., Stern, S., McGavack, T. H., *Anal. Chem.,* 1953, v27, 260.
8. Friedman, M., Rosenman, R. H., *Circulation,* 1957, v16, 339.

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Hormonal Regulation of Pituitary Adrenocorticotrophin.* (23677)

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Most of the information available concerning regulation of pituitary secretion of adrenocorticotrophin (ACTH) has been derived from indirect indices of pituitary function, primarily depletion of adrenal ascorbic acid in the intact rat. On the other hand, direct determinations of the ACTH content of pituitary tissue under various experimental conditions have been reported infrequently, and, when attempted, have often been conflicting or inconclusive(1-11).

Recently, Saffran and Schally(12) have described a technic of biological assay for ACTH based upon steroidogenesis by adrenal slices *in vitro.* Birmingham *et al.*(13) have reported a new method of extraction of pituitary ACTH. Assay of their extracts by the *in vitro* technic yielded values considerably higher than those obtained previously. These latter procedures have been employed in the following study, which was undertaken to define more clearly the effects of adrenalectomy and injection of ACTH, epinephrine, and cortisone upon pituitary ACTH content.

Materials and methods. All animals were kept in a constant temperature room for at least one week prior to use and maintained on a diet consisting of Purina Laboratory Chow and water *ad libitum.* Adrenalecto-

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TABLE I. Frequency Distribution of Index of Precision (Lambda*) for 100 Bioassays of ACTH.

Lambda	.01–.05	.05–.10	.10–.15	.15–.20	.20–.25	.25–.30	.30–.35	.35–.40	.40–.45
Frequency	1	21	30	26	6	9	5	1	1

* Lambda = assay error/slope.

mized rats received a supplement of 1% saline and 5% glucose in the drinking water. Male rats, 120-140 g, of the Sherman strain, were randomly divided into 6 groups and treated as follows: 1) 0.9% saline, 0.2 ml, injected s.c. once daily; 2) ACTH gel, 4 U.S.P. units in 0.1 ml, injected s.c. once daily; 3) cortisone acetate, 5 mg in 0.2 ml, injected s.c. once daily; 4) epinephrine in oil, 1:500, 0.1 ml, injected s.c. twice daily; 5) untreated; and 6) adrenalectomy without further treatment thereafter. The animals were injected for 7 days and sacrificed under Nembutal anesthesia 24 hours after the last injection. Body weights and weights of the adenohypophysis and the adrenal glands were measured. The individual pituitary glands were promptly extracted by the "modified glacial acetic acid method" of Birmingham *et al.*(13). Each extract was assayed for ACTH by a modification of the *in vitro* technic described by Saffran and Schally(12). The adrenal glands from each of 8 female rats, 120-140 g, were removed, cleaned and cut into eighths§ with razor blades. The 16 pieces from each rat were distributed randomly among 16 squares drawn on moistened filter paper. The 8 pieces in each square were weighed together, transferred to 25 ml Erlenmeyer flasks each containing 2 ml of Krebs-Ringers-Bicarbonate solution (KRB) made up with 1.83% rather than 1.22% $CaCl_2$(14). The tissue was incubated with shaking for 30 minutes at 37°C under an atmosphere of 95% O_2 and 5% CO_2. The medium was removed by suction and the pieces washed with 1 ml of KRB. The wash solution was discarded and 2 ml of fresh KRB added to each flask. The assay design then permitted a standard ACTH and each of 3 pituitary extracts to be added to the flasks at 2 dose levels in duplicate. The flasks were incubated for 2 hours under the conditions already described. The medium was transferred to 15 ml glass-stoppered centrifuge tubes each containing 2.5 ml of methylene chloride. The adrenal pieces were washed with 0.5 ml of KRB and the washings added to the original medium. The tubes were shaken 200 times and centrifuged at 3500 rpm for 2 minutes. A 2 ml aliquot of the methylene chloride layer was evaporated to dryness under air in round-bottomed tubes, 1 ml of absolute redistilled methanol was added and UV absorption at 225, 240, and 255 mμ measured in a Beckman DU spectrophotometer against a blank extracted from KRB alone. The Allen correction(15) was applied to the peak reading at 240 mμ and the data analyzed according to the methods of Bliss (16).

Results. One hundred bioassays of ACTH employing the *in vitro* technic have been performed. The index of precision (lambda) of each of these assays, defined as the ratio of each assay error to its corresponding slope, is presented in Table I. The median falls within the range between 0.10 and 0.15 and is comparable to values reported by authors who have employed a similar technic(12,14,17) and to those obtained by other methods. Maintenance of the animal quarters at a constant temperature was found to be the most important variable affecting assay precision. Even minor and transient variations in temperature were associated with a significant loss of precision; the values of lambda greater than 0.25 were obtained before this observation was fully appreciated.

The log dose-response curve under the conditions of the assay was demonstrated to be a straight line between doses of 8 milliunits (mu) and 256 mu of ACTH per 100 mg adrenal weight. Both the doses of standard ACTH and the assumed doses of each pituitary extract were selected within this range. Eight assays were rejected because their combined

§ Recently, the capacity of the assay has been enlarged by cutting the adrenals in twelfths with distribution in 24 flasks, thus permitting simultaneous assay of a standard and 5 unknown preparations, without loss of precision.

TABLE II. Comparison of *In Vitro* Bioassay to Ascorbic Acid Depletion Technic and Results of Repeated Assays.

Preparation	Label potency	Measured potency No. 1	95% confidence limits	Measured potency No. 2	95% confidence limits
Organon (No. 9560)	42.8 u/mg*	36.6 u/mg	19 – 54		
" (No. 51IR)	550 mu/mg†	546 mu/mg	235 –754		
Armour (commercial)	1 u/mg*	1.1 u/mg	.6– 2.7	1.0 u/mg	0.5– 2.3
Rat pituitary extract		75 mu/mg	38 –142	72 mu/mg	36 –135

* Assayed by ascorbic acid depletion technic.
† Assayed by the Saffran and Schally technic.

slopes were not significant at the 1% level of confidence. No assay required rejection owing to significant non-parallelism among preparations, although, occasionally, a single unknown was so rejected and re-assayed on the following day.

The validity and reliability of the assay technic were determined by comparison of the potencies of several ACTH preparations to those obtained by the ascorbic acid-depletion method and by re-assay of the same unknown. The results summarized in Table II, indicate both satisfactory validity and reliability with a maximum error of 15%.

The results of the bioassays of pituitary glands of the 56 animals subjected to the various experimental conditions included in the present study are summarized in Table III. Both the mean ACTH concentration (mu/mg of wet pituitary tissue) and the mean ACTH content (mu per gland) are presented for each treatment group. The ACTH values represent semi-weighted means and their approximate standard errors (Antilog $\overline{M} \pm 2.303\, s_{\overline{M}}$ Antilog $\overline{M}$) (16). The actual statistical comparisons by t test were based upon the mean log potencies and their exact log errors ($\overline{M} \pm s_{\overline{M}}$) for each group. Rigorous comparisons were ensured by derivation of the number of degrees of freedom from the number of M's in each group rather than the number available in each s_M. Each treatment group was compared with the saline-injected control group, with the exception of the adrenalectomized group which was compared with the untreated control group, since the adrenalectomized animals received no injections.

Significant and comparable depletions in pituitary concentration and content of ACTH were obtained after injection of either cortisone or epinephrine for a period of 7 days. The fall in ACTH in both these groups exceeds 60 percent when compared to the saline-injected control group. As anticipated, cortisone administration was associated with a statistically significant decrease in adrenal weight, whereas epinephrine injection was followed by significant adrenal hypertrophy.

ACTH administration, on the other hand, produced a significant increase in both pituitary concentration and content of ACTH. The concentration was almost double that obtained for the saline-treated group and content was 1.67 times greater. Adrenal hypertrophy was

TABLE III. Effects of Various Treatments on Pituitary ACTH Concentration and Content, Pituitary Weight and Combined Adrenal Weight.

Treatment group	No. of animals	ACTH conc. (mu/mg)*	ACTH content (mu)*	Pituitary wt (mg)†	Adrenal wt (mg)†‡
Saline (control)	9	41 ± 6	224 ± 35	5.6 ± .3	31 ± 1 (10)
ACTH	9	80 ± 6§	364 ± 26‖	5.1 ± .4	41 ± 1§ (5)
Cortisone	9	17 ± 3§	66 ± 20§	4.8 ± .3	20 ± 2§ (6)
Epinephrine	9	16 ± 2§	91 ± 13§	5.5 ± .1	36 ± 1§ (8)
Untreated (control)	13	63 ±12	319 ± 49	5.1 ± .4	
Adrenalectomy	7	102 ± 9¶	749 ± 60§	7.0 ± .2§	

* Antilog $\overline{M} \pm 2.303\, s_{\overline{M}}$ (Antilog $\overline{M}$). † Mean ± stand. error of mean. ‡ No. of animals studied indicated in parentheses.
§ Significant at the 1% level of confidence.
‖ Significant at the 3% level of confidence.
¶ Significant at the 5% level of confidence.

observed also, consistent with the dose of ACTH employed. The pituitary weights of the group treated with ACTH, cortisone or epinephrine did not differ significantly from those of the control group injected with saline.

Adrenalectomy resulted in a barely significant increase in pituitary ACTH concentration; however, a highly significant increase in total ACTH content was observed, well over double that of the untreated control group. The marked pituitary hypertrophy observed after adrenalectomy contributed to this increase in ACTH content. Although the mean pituitary ACTH content of animals treated with saline appeared to be 30% lower than the mean content in untreated animals, this difference was not statistically significant. Finally, the significance of all the comparisons described was unaltered when variations in body weight were taken into account.

Discussion. Measurements of pituitary ACTH concentration and content, such as those performed in the present study, permit varying interpretations. For example, increased content and concentration of ACTH may mean increased ACTH synthesis or increased storage or both. Conversely, decreased concentration or content may indicate decreased ACTH formation or decreased storage, *i.e.*, increased ACTH secretion. The following discussion must be viewed with these possibilities in mind.

The ACTH content of the pituitary glands of untreated rats as determined in the present study is significantly less than that reported by Birmingham *et al.*(13) who employed an identical procedure for extraction and a similar bioassay technic. This difference cannot be explained on the basis of the available evidence, but may be due, in part, to differences in the strain of rat studied (unspecified by the previous authors). However, the values reported significantly exceed those obtained by other methods of extraction and assay(3,11, 18).

Saline injection apparently resulted in a 30% fall in pituitary ACTH content when compared with uninjected control animals, but this difference was not statistically significant. However, the data suggest that repeated injections *per se* may result in moderate pituitary ACTH depletion; the study of more animals may be required to establish this difference.

The observation that adrenalectomy is followed by a significant rise in pituitary ACTH content associated with pituitary hypertrophy confirms previous reports(3,11) and is consistent with the hypothesis that a decrease in the circulating level of adrenal steroids is followed by accelerated production of ACTH. Conversely, an increased steroid level, produced by cortisone administration, was followed by depletion of pituitary ACTH. The associated adrenal atrophy suggests that ACTH secretion was also decreased. These findings suggest that cortisone administration for 7 days reduced ACTH synthesis with concomitant diminution of ACTH secretion. They are in accord with the report of Farrell and Laqueur (1) that cortisone injection is followed by pituitary ACTH depletion in dogs.

Epinephrine administration for 7 days is associated with significant depletion of pituitary ACTH similar to that obtained with cortisone. However, adrenal hypertrophy, presumably due to increased ACTH secretion, also was observed in contrast to the cortisone-induced adrenal atrophy. No statement concerning the effect of epinephrine upon the rate of ACTH synthesis can be made with the data currently available.

The significant rise in pituitary ACTH content following ACTH administration is surprising. The expected finding would have been pituitary ACTH depletion similar to that found in the cortisone-treated animals, since the only commonly accepted effect of ACTH is stimulation of the adrenal cortex. The increase observed is of such magnitude that it cannot be explained by the possibility of contamination of the extracted pituitary glands with blood containing even large amounts of ACTH. Experiments in progress involving administration of ACTH to adrenalectomized rats indicate that this increase in pituitary ACTH is not mediated by the adrenal gland. At least 3 alternative explanations are suggested: 1) increased blood ACTH may inhibit pituitary ACTH release; 2) an increased circulating level of ACTH may stimulate ACTH synthesis; or 3) the pituitary

gland adsorbs ACTH when present in the blood in increased concentration. Gemzell(5) has claimed that administration of ACTH for one week to adrenalectomized rats was followed by no change in pituitary ACTH content. His results are not comparable to those herein described since his technics(3) of pituitary ACTH extraction and assay yielded values 80% lower than those obtained in the present report.

Summary. A modification of the Saffran and Schally technic of *in vitro* bioassay of ACTH is described and evidence is presented for its precision, validity and reliability. Changes in pituitary ACTH content and concentration in rats after administration of various hormones for one week were studied by means of this technic. ACTH administration and adrenalectomy both resulted in a striking increase in pituitary ACTH content. Epinephrine and cortisone administration were both followed by significant depletion of pituitary ACTH.

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1. Farrell, G. L., Laqueur, G., *Endocrinology,* 1955, v56, 471.

2. Fornaroli, P., Koller, M., de Giuseppe, L., *Boll. Soc. ital. biol. sper.,* 1954, v30, 574.

3. Gemzell, C. A., Van Dyke, D. C., Tobias, C. A., Evans, H. M., *Endocrinology,* 1951, v49, 325.

4. Gemzell, C. A. *ibid.,* 1952, v50, 399.

5. Gemzell, C. A., Heijkenskjöld, F., *Acta endocrinol.,* Copenh., 1957, v24, 249.

6. Hess, M., Slade, I. H., Ammons, J. C., Hendrix, V., *Am. J. Physiol.,* 1955, v183, 261.

7. Jailer, J. W., *Endocrinology,* 1951, v49, 826.

8. McCann, S. M., Sydnor, K. L., PROC. SOC. EXP. BIOL. AND MED., 1954, v87, 369.

9. Mialhe-Voloss, C., *C. rend. Acad. sc.,* 1955, v241, 105.

10. Sayers, G., Cheng, C., PROC. SOC. EXP. BIOL. AND MED., 1949, v70, 61.

11. Sydnor, K. L., Sayers, G., *Endocrinology,* 1954, v55, 621.

12. Saffran, M., Schally, A. V., *ibid.,* 1955, v56, 523.

13. Birmingham, M. K., Kurents, E., Rochefort, G. J., Saffran, M., Schally, A. V., *ibid.,* 1956, v59, 677.

14. McKerns, K. W., Nordstrand, E., *Canad. J. Biochem. and Physiol.,* 1955, v33, 209.

15. Allen, W. M., *J. Clin. Endocr. and Metab.,* 1950, v10, 71.

16. Bliss, C. I., *The Statistics of Biossay,* Academic Press, New York, 1952.

17. Van der Vies, J., *Acta Physiol. et Pharmacol. Neerl.,* 1956, v5, 251.

18. Burns, T. W., Merkin, M., Sayers, M. A., Sayers, G., *Endocrinology,* 1949, v44, 439.

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Demonstration of Differences Between Normal and Tumor-Bearing Animals.* (23678)

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If it is assumed that tumors are metabolically or otherwise different from the normal host tissue, this distinction should express itself qualitatively or quantitatively in some of the intermediate or terminal biochemical products of malignant growth. A simple experimental design has been employed to demonstrate such differences in animals bearing transplanted tumors of various types in comparison with otherwise identical normal control animals. The principle of the procedure involves the challenging of tumor-bearing and normal mice with an intraperitoneal lethal dose of an appropriate compound capable of reacting *in vivo* with one or more of the unique metabolites associated with the tumor in question. The effect of this reaction on the survival times of the 2 groups of animals is then determined. Dissimilarity in survival times is presumably due to a decrease or in-