after 6 hours. Adrenalectomy at the time of implantation exerted some influence upon the extent of incorporation into some of the fractions, but administration of hydrocortisone for 2 days to either intact or adrenalectomized animals had no effect. When studies of glycine uptake by sponge-biopsy connective tissue proteins were made in rats of various ages, a steady decrease in rate of incorporation into all fractions was observed as age of the host increased from one month to one year.

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Failure of Hypophysectomy, Adrenalectomy or Thyroidectomy to Affect Response of Non-Esterified Fatty Acids to Fasting. (25930)

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Recent investigations focused attention upon the role of non-esterified fatty acids (NEFA) in energy metabolism. As a consequence of rapid turnover of this fat fraction, a flexible source of energy is made available to the organism(1). The plasma level of NEFA appears to be the resultant of rates of release from adipose tissue and rates of subsequent transport and utilization. The mechanisms which control NEFA release are imperfectly understood, though hormonal control and dietary factors have been implicated. Insulin inhibits(2) while epinephrine(3), growth hormone (G.H.)(4), and adrenocorticotropic hormone (ACTH)(5) accelerate release of NEFA from adipose tissue. All these substances are active *in vitro* when incubated with epididymal fat pads. Except for ACTH, they have also been demonstrated active in the intact animal. Dietary factors also influence plasma NEFA levels. Prolonged fasting results in increased levels which are decreased again following carbohydrate meal(6,2). In addition, increased *in vitro* release noted in adipose tissue obtained from fasting rats is decreased by adrenalectomy or hypophysectomy(7,4). These observations suggested that response of NEFA to diet is dependent upon hormonal control. The present study was undertaken to delineate the role of certain hormonal factors on *in vivo* response of plasma NEFA to fasting.

Materials and methods. Sprague-Dawley rats weighing 150-200 g were used. Hypophysectomized rats were obtained from Hormone Assay Lab. Purina chow diet and bread supplements were provided. Studies were performed approximately 2 weeks following surgery. Adrenalectomy and thyroidectomy were performed under ether anesthesia. Sham operation, simulating adrenalectomy was also performed. Animals were studied 5-8 days after operation. Adrenalectomized rats received 1% saline in place of drinking water. All animals, unless otherwise stated, were fed standard Purina chow and provided water *ad*

TABLE I. Response of Plasma NEFA to Fasting.

Animal preparation	No.	Fed *ad lib*	No.	16° fasted
Normal	41	380 ± 18.0*	11	957.5 ± 54.8
Controls	13	320		
Sham-operated	5	335		
Hypophysectomized	11	186.5 ± 16.2	8	744.8 ± 21.9
Adrenalectomized	10	226.8 ± 16.1	10	698.3 ± 38.3
Thyroidectomized	12	190.3 ± 27.1	12	674.8 ± 31.0

* NEFA in μeq/l ± S.E.M.

lib. Animals were ultimately sacrificed by decapitation. Blood was collected in heparinized tubes and centrifuged immediately at 4°C. NEFA was determined by adaptation of Dole's method(2) using 0.2 ml aliquots of plasma. Determinations were done in duplicate or triplicate.

Results. NEFA response to fasting. Forty-one normal rats, provided with food *ad lib.*, were sacrificed at various times during the day, to determine whether diurnal variation in NEFA occurs in rats. No significant difference in NEFA levels was found in rats killed between 8 a. m. and 3 p. m. An average figure of 380.5 μeq/l ± 18.0* was taken as normal plasma value for rats fed *ad lib.* The effect of varying periods of fasting was tested in 29 rats. Eleven animals fasted overnight for 16 hours, showed a mean value of 957.5 μeq/l ± 54.8. Three groups of 6 rats, fasted for 24, 48, and 72 hours respectively, showed the following NEFA levels: 846.0, 749.8 and 1004.3 μeq/l. Because of the highly significant difference in NEFA levels at 16 hours of fasting between fed and fasted groups ($p<.001$) this period of fast was chosen for study in various endocrine preparations which follow.

The effect of sham surgery hypophysectomy, adrenalectomy and thyroidectomy was studied. The results may be seen in Table I.

Examination of data by method of analysis of variance revealed that overall effect of fasting in all groups tested is highly significant ($p<.001$). In fasting groups, normal plasma NEFA is significantly higher than that in 3 treatment groups ($p<.001$) but the differences among the treatment groups are not significant. In feeding experiments, normal levels are significantly higher than those in treatment groups, though no differences exist among the treatments. No differences could be found in response to fasting in treatment groups as compared with normal.

* Standard error of mean.

Discussion. Our data demonstrate that hypophysectomy, adrenalectomy, or thyroidectomy results in significantly lower than normal plasma NEFA levels whether animals are fed or fasted. Despite the lower level in the fed state, the rise in plasma NEFA after fasting is of the same absolute order of magnitude as in normal animals. There was an absolute rise of 577 μeq/l in normal compared with 558, 471, and 485 μeq/l in 3 treatment groups. The per cent rise in treatment groups was significantly greater than in normal. Sham operation had no effect on NEFA levels.

These observations suggest that lower NEFA levels in endocrine operated animals are the result of deficiencies of hormonal factors. The rise in NEFA with fasting appears independent of pituitary, adrenal, or thyroid control.

It is reasonable that deficiency of G.H. and other pituitary trophic hormones such as ACTH and TSH which accelerate NEFA release might result in a lower level of plasma NEFA in hypophysectomized rats. In the thyroidectomized group, absence of thyroid hormone could explain a low NEFA level since thyroidectomy results in decreased pituitary G.H.(8), an impaired NEFA response to epinephrine(9), and thyrotoxic individuals show elevated NEFA levels(10). The effect of adrenalectomy is more difficult to explain in view of the probability that there is an increase in ACTH which accompanies this operation which might increase NEFA levels. Absence of adrenal steroids does not appear to account for low NEFA levels (unpublished observation) although absence of the adrenal medulla may be significant.

It is possible that normal NEFA response to fasting is due to the fact that decreased insulin secretion occurs with fasting. This may

then be reflected in decreased utilization of glucose by adipose tissue with subsequent increase in NEFA release. This series of changes may be independent of extra-pancreatic hormonal control.

Summary. There is no evidence of diurnal variation of plasma NEFA levels in rats. Hypophysectomized, adrenalectomized and thyroidectomized animals show significantly lower plasma NEFA values than normal rats either when food is provided *ad lib.* or after fasting. After 16 hour fast, both normal and operated rats show marked increase in plasma NEFA levels. It is probable that the mechanism involved in NEFA response to fasting is not controlled by pituitary, thyroid or adrenal hormones.

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Method for Determination of Phenylalanine Mustard and Related Alkylating Agents in Blood.* (25931)

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The growing interest in regional chemotherapy by perfusion wherein high concentrations of cytotoxic agents are employed makes it desirable to study reaction sites and persistence of these compounds in blood. A colorimetric procedure for p-di-(2-chloroethyl)-phenylalanine† (PAM) and related alkylating agents in blood has been adapted from procedure described by Epstein(1) for estimation of alkylating agents in water and non-aqueous solvents with nitrobenzylpyridine (NBP). Optimal conditions for determination of PAM were established and the revised procedure was also used for determination of HN2 and 5-(bis(2-chloroethyl)amino) uracil‡ (U-8344). The results of these *in vitro* studies on colorimetric estimation of these alkylating agents in the presence of blood are described.

Methods. Heparinized human blood with added PAM was incubated at 37°C in a water bath. Aliquots were withdrawn at intervals, centrifuged, and the plasma collected. It was noted that blood samples containing PAM could be kept at ice bath temperature for at least 3 hours without loss of color reactivity. Solutions of PAM containing less than 1 mg/ml were made in physiological saline. More concentrated solutions were made with propylene glycol and saline or with a 25% solution of ethanol in saline. In each case a slurry was made of the PAM with a few drops of the first named solvent before the major portion of solvent was added. The mixture was warmed to 40°C in water bath to complete solution, then diluted to final volume. HN2 (Mustargen, Merck Sharp & Dohme) was dissolved in water and diluted with saline. U-8344 was dissolved and diluted in 25% ethanol in saline. The reagents used for color determination were: γ-(4-nitrobenzyl) pyridine, 5% in methyl ethyl ketone,

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