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 Aliquots were withdrawn at intervals, centrifuged, and the plasma collected.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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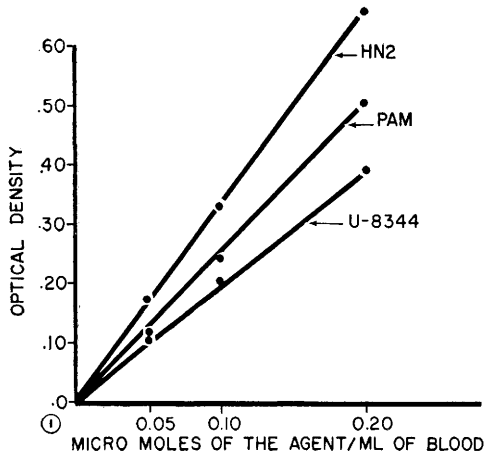

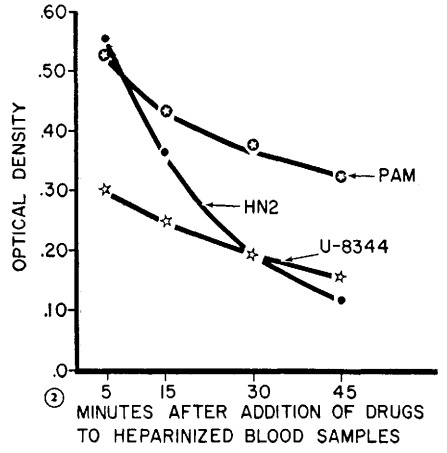

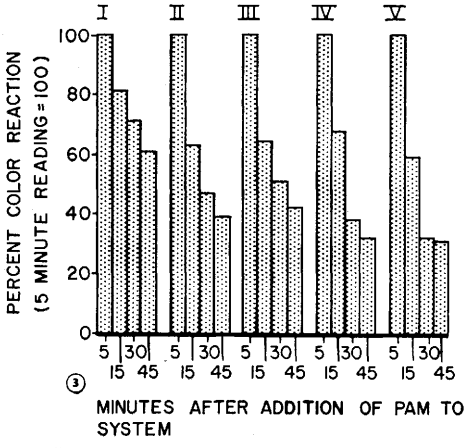

FIG. 1. Standard curves obtained by addition of NBP to alkylating drugs, PAM, HN2 or U-8344.

FIG. 2. Reaction of PAM, HN2 and U-8344 with blood as measured by color reaction with NBP. Concentration: 0.2 μmole/ml.

FIG. 3. Loss of reactive PAM from perfusion system as measured by color reaction with NBP. I. Control. PAM in heparinized blood incubated *in vitro* at 37°C. II and III. PAM in perfusion systems in patients wherein leakage was minimal. IV and V. PAM in perfusion systems in patients wherein leakage was from 25% to 30%.

triethylamine, 50% in acetone, and acetic acid, 5%. Stock solutions of PAM were made up in saline by diluting accurately weighed quantities in volumetric flasks. Aliquots were further diluted to make stock solutions to contain 0 to 0.2 μmoles/ml. One ml samples of plasma were pipetted into 12 ml centrifuge tubes followed by addition of 0.1 ml acetic acid, 2.9 ml distilled water and 0.5 ml of NBP reagent. Each tube was shaken vigorously, covered with small short-stemmed funnel, and heated 15 minutes in 80°C water bath. Tubes were then cooled in ice bath and centrifuged. Two ml of supernatant layer were transferred to a cuvette. Two ml of triethylamine color reagent were added and the sample read immediately in Bausch and Lomb Spectronic 20 colorimeter at 565 mμ employing a water blank. Absorbance of a reagent blank was determined as above and subtracted from that of sample. Amount of PAM was determined by comparing with a curve (Fig. 1) prepared by using standard PAM solutions in plasma in above procedure. The purple color formed by the reaction between PAM and NBP had a maximum absorbance of 562 mμ. For maximum color the reaction mixture should be between pH 4.5 and pH 5.0 before heating. The pH was adjusted with acetic acid. For comparison of samples, reaction mixtures should have identical pH values. The 80°C temperature was adopted in preference to boiling water because it caused less bumping of material in the reaction tube and prevented loss of sample. After heating and centrifugation, samples can be kept at room temperature for at least 1 hour without loss of color. However, measurement of absorbance immediately after addition of color reagent is imperative, as the color fades on standing. If conditions of procedure are maintained good reproducibility of absorbances is obtained.

Results. PAM was added to blood and color determinations were made at intervals (Fig. 2). A decrease of color reaction indi-

cated that PAM reacted with a component or components of blood and was then not available for color reaction with NBP. That the colored material was formed with biologically active PAM was indicated by experiments with mice and dogs (unpublished) in which PAM was dissolved in animal's blood and reinjected at intervals. White blood cell count depression decreased with decrease of colored material formed.

The reaction of PAM, HN2, and U-8344 with blood in concentrations and at conditions comparable to those in the perfusion system as measured by color reaction is given in Fig. 2. These results show that PAM and HN2, 2 agents currently used in perfusions, react with blood at different rates and persist in blood for different periods of time, while U-8344 reacts at a rate similar to that of PAM.

Loss of color reactive PAM from blood of a perfusion system may be attributed to PAM which reacted with blood of the system and with tissues of perfused part plus amount lost *via* leakage into the general circulation (Fig. 3). The loss due to reaction with blood was rapid for about 15 minutes, then proceeded at a steady rate. In a perfusion system when PAM reacted with both blood and tissues the initial loss was greater, but the general pattern was maintained when leakage was less than 5%. When leakage was of the order of 25% the pattern changed. The color reaction 5 minutes after addition of PAM to the system (5 minutes are required for thorough mixing) corresponds to less than total amount injected. Therefore, when percentage of color reactive PAM lost from the perfusion system was calculated from residual percentage (Fig. 3,V) it was actually greater than the 70% indicated by the graph.

Summary. A procedure for estimation of PAM and related alkylating agents in blood by its reaction with NBP is given. This procedure is applicable for study of the reaction with and persistence in blood of these agents *in vitro* and in perfusion systems.

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Epithelial Changes in Vaginal Isografts in Male Mice.* (25932)

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Vaginal isografts in female mice respond normally to both intrinsic and extrinsic estrogens, although response is less regular than that of the intact organ(1). Similar isografts in male mice show cornification in response to extrinsic estrogens and mucification in response to intrinsic androgens(2,3). In castrated male mice bearing ovarian transplants, cornification of vaginal grafts occurred but cyclic activity was not demonstrated. In females, similarly prepared, however, cyclic changes have been described(3). Ovarian isografts in castrated female mice or rats show cyclic formation of vesicular and luteinized follicles but do not do so in intact or castrated males(4,5). The following report concerns our observations on periods of estrus in vaginal epithelium transplanted to castrated male mice bearing ovarian isografts.

Material and methods. Intact vaginas, removed from 16- to 20-day-old strain A female mice, were transplanted into 6 groups of hosts, each consisting of 8 male strain A mice 2 to 4 months old (Table I). Three of these groups (I,II,III) were castrated at the same time and 2 of them (I,II) received bilateral intraocular isografts of one-eighth of an adult ovary from mice 2 to 4 months old, 5 to 6 weeks later. Identical ovarian transplants were made into one (IV) of the 3 non-castrated groups (IV, V, VI). All vaginal grafts were fistulated so that smears of exfoliated

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