necessary for this response. It is concluded that maximum LNPSH depletion during cold and restraint can be effected through stimulation of the sympathetic nervous system although other mechanisms are operative in intact animals.

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Evidence for Omitting the Petroleum Ether Extraction in Plasma Corticosterone Determination.* (31722)

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The original method for spectrophotofluorimetric determination of serum corticoids(1) and its modification(2) suggest that the sample be extracted with either pertoleum ether or isooctane. This step removes neutral lipids(3) which would interfere with the analysis of the corticoids. However, the studies of Callard *et al*(4) indicate that 20 α-hydroxy-pregn-4-en-3-one (hereafter called 20 α-hydroxyprogesterone) may be the main interfering fluorogen in determination of plasma corticosteroid levels. This compound has absorption and fluorescent spectra which are similar to corticosterone(4). Twenty α-hydroxyprogesterone has been found in the plasma and ovary of pregnant female rats(5) and in the venous effluent of the ovary in normal female rats(6). About half of this compound is removed from plasma by an extraction with petroleum ether (B.P. 40-60°) (4).

If only the ovary produces 20 α-hydroxyprogesterone and if it is the main source of error in the fluorimetric method for corticoid determination, then the extraction with petroleum ether may be unnecessary for the analysis of samples from male and ovariectomized female rats. The following experiments were designed to test this hypothesis.

Methods and materials. Fifty-nine Sprague-Dawley rats were sacrificed by guillotine or under ether. The blood was collected in heparinized containers and centrifuged at 1000 × *g*. The plasma was removed and frozen.

Of these 59 rats, 9 were males, intact or hypophysectomized. Nineteen were females which had been ovariectomized 2 weeks prior to use. Thirty-one animals were intact females which were maintained in a light-regu-

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TABLE I. Effect of Petroleum Ether (PE) Extraction(s) on Fluorescent Levels at 535 mμ in Plasma Samples.

Type of preparation	No. of rats	No PE extraction	1 PE extraction	P value†	No. of rats	2 PE extractions	P value‡
Female rats							
Proestrus	8	48.6 (21.5-73.0)*	40.6 (19.5-72.0)	<.01	6	46.6 (21.8-74.8)	>.10
Estrus	10	38.2 (8.9-63.7)	38.0 (7.4-56.5)	<.05	8	27.7 (8.0-45.6)	>.10
Diestrus	13	30.8 (3.9-61.1)	31.1 (4.1-42.6)	<.01	10	25.7 (3.6-42.1)	=.02
Ovariectomized	19	7.1 (3.6-93.3)	7.4 (2.5-93.5)	>.05			
Male rats							
Intact and hypophysectomized	9	27.5 (3.5-46.2)	26.7 (4.7-46.1)	>.10			

* Fluorescent levels in μg % plasma. Median and range given.
† P value between samples receiving 1 and samples receiving no PE extraction.
‡ P value between samples receiving 1 and samples receiving 2 PE extractions.

lated, temperature-controlled room for at least 4 weeks. Vaginal smears were obtained on this last group 2 hours before they were sacrificed. The smears were stained with 1% thionin and classified as proestrus, estrus, or diestrus by microcopic observation.

Plasma corticosterone levels were determined by the method of Silber *et al*(1), as modified by Gillemain *et al*(2) in an Aminco-Bowman Spectrophotofluorometer at an activating wavelength of 475 mμ and a fluorescing wavelength of 535 mμ. Duplicate one-half ml portions of each sample were treated identically, except that one aliquot from each animal received no petroleum ether (B.P. 60-80°) extraction. In addition, a third aliquot of plasma from each intact female rat was extracted twice with petroleum ether. Thus, each animal served as its own control. The resulting data were analyzed by the Wilcoxon matched-pairs signed-ranks method(7, 8),

Results. Table I presents a comparison of fluorescent levels at 535 mμ in plasma samples. All samples from intact female rats were divided into 3 aliquots. One aliquot received no extraction with petroleum ether; the second, one extraction; and the third received 2 extractions with petroleum ether. The samples are grouped according to the stage of the estrous cycle exhibited by the rat on the day of sacrifice. Statistical evaluation was carried out a) between samples receiving no extraction with petroleum ether and one extraction and b) between samples receiving one and two extractions. Significant decreases in fluorescence were found between aliquots receiving one extraction with petroleum ether, when compared to portions receiving no extraction, when the blood was obtained from rats in proestrus ($P<0.01$), estrus ($P<0.05$), or diestrus ($P<0.01$). When a second extraction was applied to a portion of the plasma, fluorescent levels were further decreased in samples from rats in diestrus ($P=0.02$). This additional step failed to produce a significant change in samples from animals in proestrus or estrus ($P>0.1$ in both groups).

The results obtained on samples from either ovariectomized females or intact and hypophysectomized males contrast to those presented for intact females. A single extraction failed to lower fluorescent levels in plasma samples from these two groups ($P>0.05$ and $P>0.10$, respectively).

Discussion. Statistically significant decreases in fluorescent levels at 535 mμ were obtained when plasma samples from intact female rats were extracted once with petroleum ether, as compared to when this step was omitted. This result occurred in samples obtained from female rats in all stages of the estrous cycle. In contrast, fluorescent values failed to change significantly when plasma from male or ovariectomized female rats received this extraction. However, 5 samples in the latter group showed decreases of about 5 μg% plasma or more after the extraction. No such variation appears in the plasma values for male rats. The maximum change in fluorescent levels for the pairs in this series is about 2 μg%. It is likely that this difference could be accounted for by the

variability in the procedure. Alterations of 5 μg% represent a difference of approximately 5 units in the fluorescent reading. This change probably represents a real difference in the samples. Thus, in some cases fluorescent levels in plasma samples from ovariectomized females show an apparently real decline after extraction with petroleum ether. Unless the half-life of 20-hydroxyprogesterone, the compound postulated to alter fluorescent levels in this assay, is about 2 weeks, this result obtained in a few ovariectomized females is probably not accounted for by ovarian secretion.

Callard *et al*(4) have reported that one extraction with petroleum ether (B.P. 40-60°) removes 56.8% of the interfering fluorogen but leaves 93.8% of the corticosterone. The data in Table I suggest that a second extraction with petroleum ether fails to alter fluorescent levels. Thus, a second extraction with petroleum ether did not produce significant changes in fluorescent levels in samples from rats in proestrus or estrus ($P>0.1$). Although this additional step produced a statistically significant change in samples from rats in diestrus ($P=0.02$), the actual change does not exceed 2.5 μg% in any pair. This small difference lies within the variability of the fluorimetric method. Perhaps the fraction with the higher boiling point (used in the present study) removes more of the 20α-hydroxyprogesterone than the fraction with the lower boiling point. This may explain the failure of a second extraction with petroleum ether to produce an additional decline in levels, compared to the reduction obtained with one extraction (Table I).

It is interesting that the extraction with petroleum ether is effective in altering fluorescent levels in plasma samples from female rats at all stages of the estrous cycle. Eto *et al*(6) have reported that 20α-hydroxyprogesterone is present in blood from the ovarian vein during all stages of the estrous cycle. Apparently, sufficient quantities are present at all times in the plasma obtained from intact female rats to interfere with the determination of corticosterone levels.

Summary. Fluorescent levels at 535 μ were measured in samples obtained from intact females, intact or hypophysectomized males, and ovariectomized females. Each sample was divided so that one part received no extraction with petroleum ether and the other portions received at least one extraction. The addition of this step failed to alter significantly the fluorescent levels in samples from male rats or ovariectomized females. In the latter group, occasional pairs of samples showed differences of at least 5 μg% plasma. In contrast, fluorescent values declined following a single extraction with petroleum ether in samples obtained from intact females at all stages of the estrous cycle. A second extraction produced only small changes (<2.5 μg% plasma).

These data support the hypothesis that the main interfering fluorogen in the fluorimetric determination of plasma levels of corticosterone is secreted by the ovary. A likely candidate is 20α-hydroxypregn-4-en-3-one. The extraction of the plasma with petroleum ether may be safely omitted in samples obtained from male rats. This reduces the time required to determine corticosterone levels.

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