and in water, due to dehydration and hydration, respectively, added further to the uncertainty of this method.

On increasing the temperature of c.s. solutions 5°F above that at which they were standardized, the specific gravity values were found to remain constant within 0.001. Even when the tissues had been stored in the ice box, equilibrium was attained so quickly when 2 mg samples were tested that no appreciable change in specific gravity could be detected.

The specific gravity of c.s. solutions used for 25-50 tests was found to remain constant within 0.001 for a week. However, the specific gravity of a solution increased after prolonged contact with the tissue and the color of the solution changed from blue to green. A periodic check of the specific gravity of the working solutions should be made with a hydrometer. The standard solutions, stored in stoppered bottles, should maintain their values indefinitely.

Conclusions. The c.s. method for determination of specific gravity of fresh tissue requires only small samples, and is precise, quick and simple to use. With proper precautions against air bubbles and loss of moisture from tissue by exposure to air, this method avoids most of the difficulties inherent with Archimedes method and is more precise. It should find application not only for determining the specific gravity of organs of experimental animals but also for rapid detection of fatty livers by means of biopsy samples(5).

Summary. The copper sulfate method of determining the specific gravity of blood has been applied to fresh tissues. A number of factors influencing the determinations have been studied. Comparison of this method with that based on Archimedes principle have shown the copper sulfate method to be decidedly superior.

1. Tsai, C., Lin, C., Chinese J. Physiol., 1939, v14, 39.

2. Saathoff, J., Monat. f. Veterinärmedizin, 1947, v2, 191.

3. Phillips, R. A., Van Slyke, D. D., Dole, V. P., Emerson, K., Hamilton, P. B., Archibald, R. M., Bumed News Letter, U. S. Navy, 1943, v1, 1.

4. Morales, M. F., Rathbun, E. N., Smith, R. E., Pace, N., J. Biol. Chem., 1945, v158, 677.

5. Billing, B. H., Conlon, H. J., Hein, D. E., J. Clin. Invest., 1953, v32, 214.

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Edema of the Skin and Menstruation in Monkeys (*Macaca mulatta*) On Repeated Estrogen Treatments.* (31051)

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The development of a sexual skin in adolescent female monkeys *(Macaca mulatta)* and its subsequent maturation into a condition seen in the adult has been described(1). The maturation process involves the loss of an edematous condition responsive to estrogen to one in which the sexual skin is comparatively thin and has a reddish color which may not only include the perineum and buttocks but also extend for various distances down the legs, upon the flanks, and over the symphysis pubis. The pale sexual skin of a castrated juvenile animal becomes greatly swollen when estrogen is given, whereas that of a castrated adult develops a red color and shows little or no edema.

When castrated adult monkeys are given large doses of estrogen a generalized edema of the skin results which may extend over most of the body with the exception of the matured sexual skin of the buttocks and perineum. The skin of the back and sides develops prominent pachydermatous folds (Fig. 1). The face also is usually affected, particularly the supraorbital ridge, and swelling about the eyes may be so great as to partly obstruct vision. Such a condition can be de-

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veloped in about 2 or 3 weeks, and if the treatment is continued for an extended period the edema tends to subside(2,3).

Progesterone has a strong inhibitory action on the production of edema by estrogen both on the sexual skin of the perineal area of sexually immature animals and the generalized response to large doses. If daily injections of progesterone are added to the treatment after a full response to estrogen has developed, a noticeable loss of edema begins on about the fourth or fifth day and swelling of the skin disappears almost completely by about the tenth day. When both estrogen and progesterone are administered concurrently to a castrated adult monkey from the beginning of an experiment edema does not develop, but the sexual skin of the perineal area regains its normal red color, an effect which either hormone can induce when given separately (Fig. 2).

These observations raised the question as to whether the skin of the body other than the perineal area would "mature" and become refractory to large doses of estrogen as indicated by a failure to show an edematous response. Also, it was of interest to determine the influence of such treatments on subsequent, estrogen-withdrawal bleeding.

Methods and results. Edema of the skin. Although observations bearing on these matters have been made in previous studies, data used in the present report were obtained mostly from experiments initiated in 1957 on 6 castrated adult monkeys weighing 4 to 5 kg, and which had not been used previously for experimental purposes. All animals were started on treatments at the same time. In the first treatment they were given daily doses of estradiol-17B or estriol in amounts sufficient to produce generalized edema of the skin. When a full response was obtained within about 3 weeks the dosage of estrogen was progressively reduced at approximately weekly intervals to determine the minimal amount of hormone required to maintain the edematous condition of the skin. Although there was considerable individual difference in responsiveness of the skin it seemed that on initial treatment 0.5 mg of estriol daily was about as effective as 0.25 mg of estradiol in

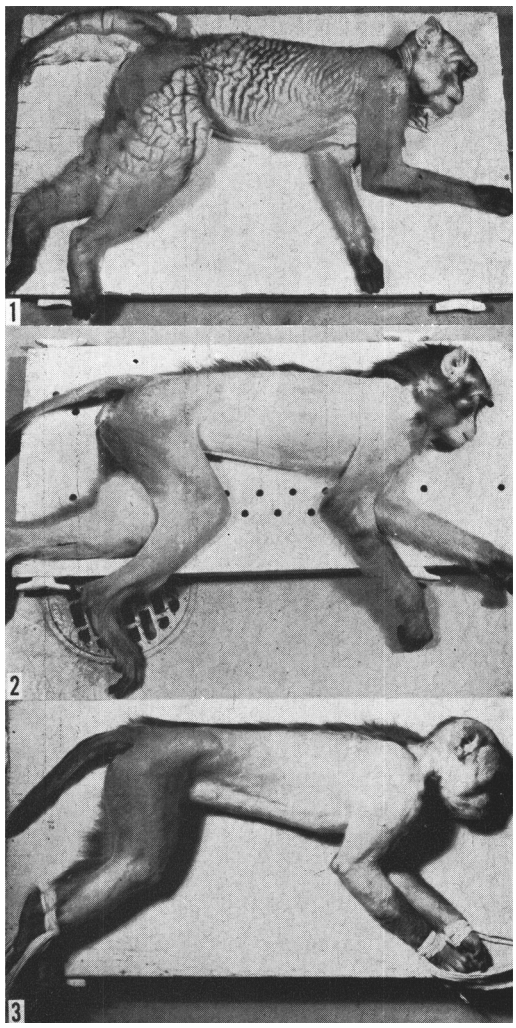

FIG. 1. Adult monkey. Castrated and given 1.0 mg estriol daily for 30 days. Note generalized edema and pachydematous folding of the skin, also swelling of supraorbital area. The matured sexual skin on the rump did not become edematous but developed the usual red color characteristic of adult animals.

FIG. 2. Adult monkey. Castrated and given 1.0 mg estriol plus 2.0 mg progesterone daily for 30 days. Progesterone prevented generalized edema of the skin but red color of the matured sexual skin on the rump was not affected.

FIG. 3. Adult monkey (251). Castrated and given 2.0 mg estriol daily for 30 days after refractoriness of the skin to estrogen had developed due to previous treatments. There is no generalized edema or wrinkling of skin. Development of red color of the mature sexual skin on the rump was retained. Response of the skin during initial treatments with 0.5 or 1.0 mg estriol daily was similar to that shown in Fig. 1. This animal also became a "non-bleeder" in that withdrawal bleeding ceased to occur following an estrogen treatment.

the production of generalized edema, and larger doses of either hormone intensified the reaction. Also, the edema produced by 0.5 mg estradiol was not maintained by 0.25 mg estriol, though that produced by 0.5 mg estriol was sustained by 0.25 mg of estradiol but not by 0.125 mg.

When marked edema of the skin was induced by large doses of estrogen daily (estradiol 0.25 mg, estriol 0.5 mg, or more) and the treatment discontinued, swelling of the skin was lost within about a fortnight. It was found that a subsequent treatment, in which the same dosage of either hormone was used, produced less edema of the skin than the previous one. The effect, however, could be intensified by increasing the dose, thus indicating that the skin was less responsive. When such experiments were repeated the skin became more and more refractory to estrogen until finally large doses caused no visible edema (Fig. 3). Although the skin in later treatments did not become edematous and pachydermatous it was nevertheless often wrinkled, indicating that response of the skin to the growth stimulus of estrogen was retained. Also, pronounced enlargement of the supraorbital area of the face continued to occur in successive estrogen treatments long after the skin of the body had become refractory.

Progesterone had a strong inhibitory effect on the response to estrogen when the two hormones were administered concurrently, which is an effect commonly observed even on initial treatments(1). This inhibitory action of progesterone in addition to protecting the skin against the edematous effects of estrogen also is more effective in hastening the appearance of refractoriness to subsequent estrogen treatments (Fig. 2).

The condition of refractoriness of the skin to estrogen once developed was retained for at least several months if not permanently. There was always a lapse of a month or more between experiments and in one instance (Monkey 250) no injections were given for over a year, March 10, 1960, to October 16, 1961; yet no improvement was observed in the response of the skin to estrogen. The skin of this monkey (250) was still nonresponsive when the animal was killed following a series of estrogen injections starting March 5, 1963. This also was true for 2 other animals (251 and 252) given similar treatments and which were continued on experiments for 2 additional years, during which they received three 30-day treatments of 2.0 mg estriol daily in 1963-'64 and one of 1.0 mg and 3 of 2.0 mg estriol daily for 30 days in 1964-'65.

Uterine bleeding. It was found in the initial experiments that if the daily dosage of either estradiol or estriol was gradually decreased, in time the treatment could be discontinued without subsequent bleeding. This, however, occurred in only 2 of the 6 animals. It may be that in some experiments the dosage was decreased too rapidly or a dosage incapable of maintaining the endometrium at a given stage of involution was continued too long and resulted in "breakthrough" bleeding. An experiment in which discontinuance of the treatment under these conditions was not followed by bleeding is shown in the accompanying Table giving data on Monkey 250, which in other respects is representative of all 6 animals with certain exceptions.

An unusual development which appeared during the course of repeated experiments was the absence of withdrawal bleeding which ordinarily follows precipitous discontinuance of a series of estrogen injections. It has been common experience that such bleeding invariably takes place within a week or 10 days even when a small daily dose of estrogen is given for a fortnight or longer. In fact, after 10 μg of estradiol has been given daily for about 40 to 50 days, bleeding may follow if the treatment is skipped for a day or so(4). Thus it appears that under these conditions dependence upon the presence of estrogen stimulation increases. In the present experiments, however, it was found that in a prolonged series of treatments a condition was established in certain animals in which bleeding may not follow the administration of comparatively large dosages of estrogen.

There was considerable variation with respect to withdrawal bleeding among the 6 monkeys on which these observations were made. Two animals became "non-bleeders," 3 occasionally failed to bleed, and one (252)

TABLE I. Effects of Estrogen and Progesterone on Edema of the Skin and Withdrawal Bleeding (Monkey 250).

Exp[1]	E_2 (mg)[2]	E_3 (mg)[3]	P (mg)[4]	Days[5]	Edema[6]	Bleeding[7]	Withdrawal[8] bleeding
1) 10- 3-57	.5			32	great	none	
11- 4-57		.25		11	decrease	"	
11-15-57		.125		14	normal	"	
11-29-57		.062		27	none	"	
12-26-57		.041		22	"	"	
1-17-58		.030		39	"	"	
2-25-58		.020		35	"	"	
4- 1-58		.015		8	"	"	
4- 9-58		.010		9	"	"	
4-18-58		.005		13	"	"	
5- 1-58		.0025		6	"	"	none
2) 9-29-58		.5		31	some	"	
10-31-58		1.0		11	much	"	not observed
3) 1-22-59		1.0	1.0	31	none	Feb 11-13	none
4) 5-11-59		1.0		29	"	none	not observed
5) 10- 1-59		1.0		39	"	"	" "
6) 12- 8-59		1.0		36	"	"	none
7) 2-14-60	.01			19			
3- 5-60	.01		2.0	5			
3-10-60	.01			50	"	"	"
8) 10-16-61		.5		30	"	"	"
9) 2- 5-62		.5		30	"	"	"
10) 4-16-62		1.0		30	"	"	"
11) 10-19-62		1.0		30	"	"	"
12) 1-12-63		1.0		4			
1-16-63		2.0		25	"	"	"
13) 3- 5-63		2.0		30	"	"	killed

1. Dates indicate beginning of a treatment and changes in dosage of hormones. 2. E_2, estradiol. 3. E_3, estriol. 4. P, progesterone. 5. Days, duration of a treatment. 6. Edema, effects on skin over the body. Wrinkling of skin without edema was recorded as negative. 7. Bleeding, "breakthrough" bleeding during course of treatment. 8. Withdrawal bleeding, bleeding subsequent to a treatment.

continued to show withdrawal bleeding though given 20 treatments, most of which were for approximately 30 days between 1957 and 1965. One of the "non-bleeders" developed the condition rather early (250) as shown in Table I and the other (251) much later (1964) after having been given 17 series of estrogen injections. That a similar condition might have developed in others had the treatments been repeated for a longer time remains problematic. Some indication that this might be the case is suggested by the fact that in most animals as the treatments were repeated the interval between estrogen withdrawal and subsequent bleeding tended to increase and in certain instances was as much as 20 days. Also, periods of actual bleeding usually became shorter and more scanty. Monkey 252, however, showed withdrawal bleeding which began on the 8th, 7th, 8th, and 13th day respectively following the last treatments (17, 18, 19, 20, 1965) in which 2.0 mg estriol was given daily for 30 days.

An additional feature shown by the 2 animals in which withdrawal bleeding ceased to occur was that this condition once established seemed to persist (see Table I for No. 250). Rest periods of 6 months or longer did not correct the condition in 250, nor did this animal bleed following treatments in which progesterone was given concurrently with estrogen. This condition of "non-bleeding" following an estrogen treatment seems to apply also to Monkey 252. Failure of the endometrium to regain normal responsiveness to estrogen, as indicated by withdrawal bleeding, resembles to this extent the persistent refractoriness of the skin of the body and the perineal sexu-

al skin to estrogen following maturation.

The biochemical nature of both the edematous reaction of the skin and the development of the endometrium in response to a series of injections of estrogen is still quite obscure. It has been found that edema of the sexual skin of the perineum involves an increase in both plasma protein(5,6) and hyaluronic acid (7). The available evidence seems to support the hypothesis suggested by Zuckerman(8) that hyaluronic acid affects the water content of the sexual skin by causing retention of serum protein in the extracellular space. Biochemical changes involved in the development of refractoriness to estrogen has not been determined.

Summary. The marked edematous effect of large doses of estrogen on the skin of monkeys decreases progressively on repeated treatments. The response finally attained is characterized by a general absence of edema though some growth and wrinkling of the skin may occur. A concomitant effect is a lengthening of the interval between discontinuance of an estrogen treatment and subsequent withdrawal bleeding, and in certain animals such bleeding may cease to occur. These conditions in the skin and uterus once established seem to persist indefinitely.

1. Hisaw, F. L., Hisaw, F. L., Jr., Sex and Internal Secretion, Williams & Wilkins, Baltimore, 1961, 556.

2. Bachman, C., Collip, J. B., Selye, H., Proc. Roy. Soc., London, ser. B, 1935, v117, 16.

3. Hartman, C. G., Geschickter, G. F., Speert, H., Anat. Rec., Suppl. 2, 1941, v79, 31.

4. Hisaw, F. L., Endocrinol., 1942, v30, 301.

5. Cohen, S., J. Endocrinol., 1955, v12, 196.

6. ———, Biochem. J., 1956, v64, 280.

7. Rienits, K. G., ibid., 1960, v74, 27.

8. Zuckerman, S., Brit. Med. Bull., 1955, v11, 111.

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Effects of Clomiphene Citrate on the Mouse Uterus.* (31052)

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Clomiphene, 1-p (β-diethylaminoethoxy) phenyl, 1-2-diphenyl-2-chloroethylene, is an analogue of the nonsteroidal estrogen, chlorotrianisene (TACE). Clomiphene has been shown in rats to have gonadotrophin inhibiting, antiovulatory, and antifecundity effects (1,2), but in the human, it has been shown to be capable of inducing ovulation in anovulatory women(3,4). Recently Roy, Greenblatt and Mahesh(5,6), have shown that clomiphene in rats has both estrogenic and antiestrogenic effects on the uterus. The uterotrophic action was observed in hypophysectomized immature female rats without any evidence for ovarian stimulation(5). The uterotrophic action of exogenous estrogen was inhibited by clomiphene(6).

Similarly, another nonsteroidal compound, ethamoxytriphetol (MER-25), has been shown to have uterotrophic effects, stimulating an increase in alkaline phosphatase enzymatic activity as well as producing uterine weight gain over a similar time course(7).

The present study was attempted to demonstrate that the uterotrophic action of clomiphene is typically estrogenic; that is, stimulating an estrogen sensitive enzyme system as well as stimulating uterine weight gain. The antiestrogenic effects of clomiphene were detected and studied.

Materials and methods. Bilateral ovariectomy was performed on immature female Swiss mice weighing 8-10 g, and they were kept on a normal diet *ad libitum* for 12 days before treatment. The mice were killed by cervical dislocation and examined at 0, 6, 12, 24, 36, 48, 72 and 96 hours after a single subcutaneous injection of either 0.1 μg estradiol dipropionate or 0.2 mg clomiphene citrate. Other groups of mice were pretreated for 3 days with 0.1 μg estradiol, 0.1 mg

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