

THE EFFECTS OF ORAL CONTRACEPTIVES
ON SEX CHROMATIN

by

James Gus Chafouleas

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SIGNED:

James Leo Chafetz

APPROVAL BY THESIS DIRECTOR

This thesis has been approved on the date shown below:

John R. Davis, MD
John R. Davis, M. D.
Associate Professor of
Pathology

25 September 1973
Date

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ABSTRACT

Sex chromatin body frequencies in four women were determined from daily buccal smears. The effects of oral contraceptives on this frequency and a cyclic fluctuation during the menstrual cycle were observed. Oral contraceptives introduce large amounts of estrogen into the system and stop ovulation through a feedback mechanism on the pituitary gland. High and consistent sex chromatin body frequencies were observed in two subjects on the oral contraceptives Norquen and Norinyl 1+80 28 day. The two subjects not taking oral contraceptives showed an increase in the sex chromatin frequency during the ovulatory period of the menstrual cycle. High sex chromatin frequencies may be related to high estrogen levels.

INTRODUCTION

Barr and Bertram (1949) first identified sex chromatin as a heteropyknotic body occurring in the nucleus of the neurons of female cats, but absent in males. Today the sex chromatin body is found in many interphase nuclei of all mammals. The sex chromatin body is observed as a planoconvex heteropyknotic 1 μ m structure adjacent to the inner surface of the nuclear membrane.

The Lyon hypothesis (1966) states that only one X chromosome is genetically active in a given nucleus and that the sex chromatin body represents an inactive X chromosome in a nucleus containing more than one X chromosome. The inactivation of this X chromosome is of a random nature and occurs in early embryonic life. Therefore, the number of sex chromatin bodies observed in a nucleus should represent one less than the total number of X chromosomes in that nucleus. Experiments using the X-linked gene for erythrocyte G-6-PD have been performed to verify this hypothesis (Riesenberq et al. 1966 and 1967). These experiments showed a direct correlation between a decrease in the number of sex chromatin body positive cells counted and an increase in the concentration of erythrocyte G-6-PD.

Mittwoch (1964) observed that the nuclei containing sex chromatin bodies were significantly smaller than those lacking them. This difference could be attributed to several reasons: (1) that the sex chromatin

are in nuclei of cells at a mitotic state where the nuclei are normally small or, (2) it is an effect of condensation of the X chromosome.

A fluctuation in sex chromatin body frequency during the menstrual cycle has been a controversial topic for many years. Many papers have been published which verify the fluctuation (Blanco de del Campo and Ramirez 1965, Schmidt et al. 1966, Miller and Warburton 1968, Dokumov and Spasov 1968, Cavelli et al. 1970, and Hagy and Brodrick 1972). Others have been published which refute it (Brainerd, Mercer and Miles 1965, Dolan 1968, Levij and Carel 1968, and Curtis 1969).

Women with breast carcinomas also exhibit a fluctuation in sex chromatin body frequency (Stanley 1966, and Seshadri, Shan and Trivedi 1970) as do those women sensitive to rag weed pollen (Platt and Kailin 1969) and burn victims (Weste et al. 1967).

The question as to whether the fluctuation of the sex chromatin body frequency is a function of the cell cycle was answered by Comings (1967) and Klinger, Schwerzacker and Weiss (1967). They demonstrated that the sex chromatin did not despiralize during DNA synthesis and that it starts its replication later and takes longer to replicate than euchromatin. Klinger et al. (1968) later demonstrated that the fluctuation in sex chromatin is not due to the cell cycle but to the cell's metabolic state.

Dokumov and Spasov (1967) demonstrated a relationship between the frequency of sex chromatin bodies and the concentration of certain sex hormones. They observed that testosterone and progesterone both caused a decrease in the sex chromatin frequency while estrogen caused a

significant increase. Becker, Martin and Boukhris (1971) have observed a decreased sex chromatin body count with progesterone and estrogen and Townsend, Case and Lucas (1970) have shown a fluctuation in sex chromatin bodies during pregnancy. Lysovitch and Margulis (1969) have observed an increased concentration of red cell G-6-PD during the secretory phases of the menstrual cycle.

Cytogenetic studies have been carried out on women using oral contraceptives (McQuarrie et al. 1970). The study dealt with the karyotypes of the mothers and their eventual offspring, but did not investigate the frequencies of sex chromatin bodies in the subjects.

It has been demonstrated that there is a fluctuation of the frequency of sex chromatin bodies during the menstrual cycle and that this fluctuation is influenced by sex hormones as well as other substances. This study therefore was designed to determine whether there definitely is a fluctuation in the frequency of sex chromatin bodies during the menstrual cycle, to observe the effect of oral contraceptives on the frequency of sex chromatin bodies during the menstrual cycle, and lastly, to set up guidelines for clinical interpretations of buccal smears.

METHODS AND MATERIALS

Buccal smears were taken from four healthy volunteer female subjects.

Subject I was a 26 year old woman who had been taking Ortho-novum 1/80 28 as an oral contraceptive from 1967 to 1971. Ortho-novum 1/80 28 is a combination type oral contraceptive manufactured by Ortho Pharmaceuticals consisting of 21 tablets containing norethindrone 1 mg. with mestranol 0.08 mg., and 7 tablets with inert ingredients. From 1971 through this study the subject used no oral contraceptives.

Subject II was a 25 year old woman who had never taken any type of oral contraceptive.

Subject III was a 22 year old woman who has been on Norquen as an oral contraceptive for the past five years. Norquen is a sequential type oral contraceptive manufactured by Syntex Laboratories with 14 tablets containing only mestranol 0.08 mg., and 6 tablets containing mestranol 0.08 mg. and norethindrone 2.0 mg.

Subject IV was a 24 year old woman who had been on the oral contraceptive Norinyl 1+80 28 day for the six month period immediately preceding the study. Norinyl 1+80 28 day is a combination type oral contraceptive manufactured by Syntex Laboratories consisting of 21 tablets containing norethindrone 1 mg. with mestranol 0.08 mg., and 7 tablets with inert ingredients. This subject remained on this oral contraceptive for the first month of the study and discontinued use for the second month.

Daily buccal smears were taken for a one month period for subjects I and II and for a two month period for subjects III and IV.

All slides were made by scraping the buccal mucosa forward several times with a metal scraper and smearing the cellular material on a frosted end microscope slide in one direction only, followed by immediate immersion into a Coplin jar containing a fixative of 95% ethanol for at least fifteen minutes. Two slides were taken from each subject on each day. The standard staining procedure of the Cytogenetics Laboratory of the Arizona Medical Center at The University of Arizona was used (Ed LAVOR, Laboratory Technician, Arizona Medical Center, personal communication, November 1972).

All slides were immersed in:

1. 70% ethanol for fifteen seconds.
2. 50% ethanol for fifteen seconds.
3. Distilled water for fifteen seconds.
4. 1% solution of cresyl violet acetate for five minutes.
5. Two separate washings of distilled water for fifteen seconds each.
6. 50% ethanol for fifteen seconds.
7. 70% ethanol for fifteen seconds.
8. Two separate Coplin jars containing 95% ethanol for two minutes each.
9. 100% ethanol for a few quick dips.
10. Xylene until ready to mount.

All slides were mounted in Permount and allowed to dry at least twenty-four hours.

The slides were then coded by a fellow graduate student and read only by myself.

Each slide was first scanned under 400X to determine cell distribution and locate random groups of cells to be read. 1,000X oil immersion was used for the actual scoring. 100 well defined oval nuclei with smooth nuclear membranes were scored as sex chromatin positive or negative per microscope slide. Only those nuclei which contained a well defined, deeply stained body adjacent to the nuclear membrane were scored as sex chromatin positive, all others were scored negative.

A graph with the coordinates % X chromatin vs. day of study was plotted for each subject after all the slides had been read.

RESULTS AND DISCUSSION

The subjects of this study can be divided into two groups: those who were off the oral contraceptives during the study (subjects I & II) and those who were on the oral contraceptive (subjects III & IV).

Subjects I and II demonstrated a great deal of daily fluctuation in sex chromatin body frequencies during the menstrual cycle. (figure 1). In interpreting the data the menstrual cycle was divided into three sections. Section A is the period starting from the first day of menses to the presumed onset of ovulation; section B is the anticipated ovulatory period; section C is from the end of the ovulatory period to the beginning of the next menses.

Subject I had a menstrual cycle of 22 days during the study. Table 1 shows that the mean of the ovulatory period is greater than the means of the pre and post-ovulatory periods. This difference between the means, however, is not statistically significant.

Subject II had a menstrual cycle of 22 days during the study. Table 1 shows that a comparison of the means of the pre and post-ovulatory periods shows them to be statistically similar. The increase in sex chromatin body frequency during the ovulatory period is statistically significant when compared to the pre and post-ovulatory periods.

Subjects III and IV (figure 2) also demonstrated some daily fluctuation in the daily frequency of sex chromatin bodies. The analysis of their data, however, is different than for the subjects off the oral

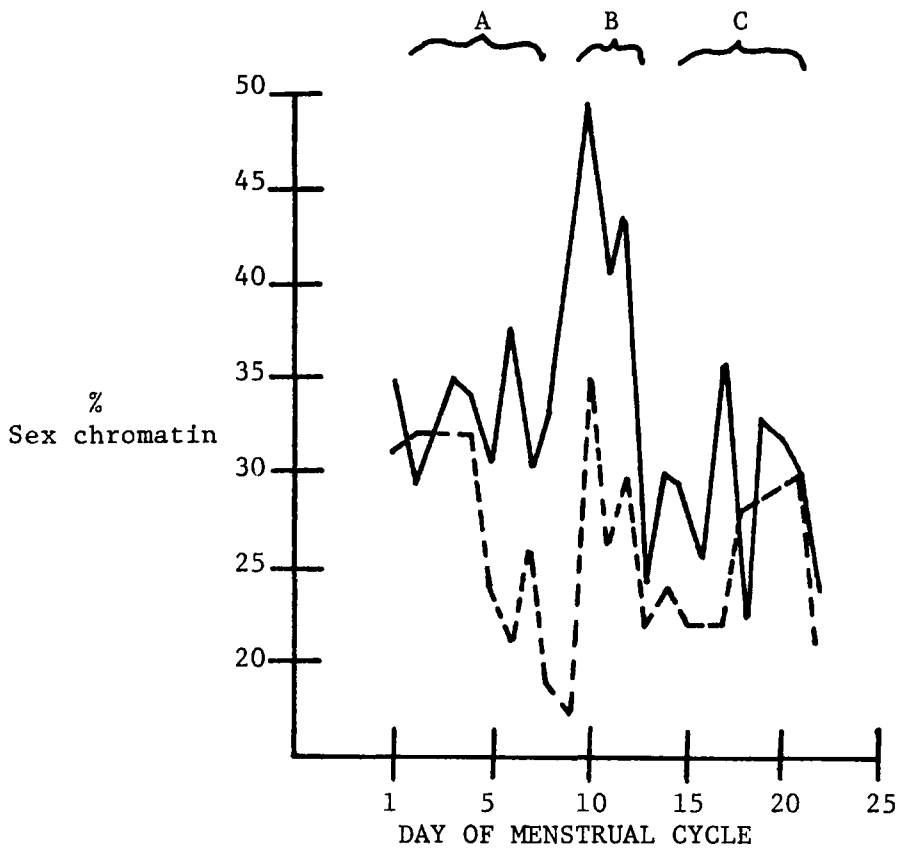


Figure 1. Daily buccal smears of the subjects not on oral contraceptives (subjects I & II).--Subject I ----- line, subject II _____ line. Bracket A is the pre-ovulatory period, bracket B is the ovulatory period, and bracket C is the post-ovulatory period.

Table 1. Statistical Analysis of Subjects off Oral Contraceptives (Subjects I & II)

Subject	\bar{X} of	\bar{X} of	\bar{X} of	Comparisons between sections		
	Pre-ovulatory Section A	Ovulatory Section B	Post-ovulatory Section C	A & B	B & C	A & C
I	26.6	30.3	26.6	t=1.1586 p<0.5	t=1.3441 p<0.5	t=0.1528 p>0.5
II	32.5	44.6	29.1	t=4.3397 p<0.0010	t=4.8087 p<0.0005	t=1.8966 p<0.1

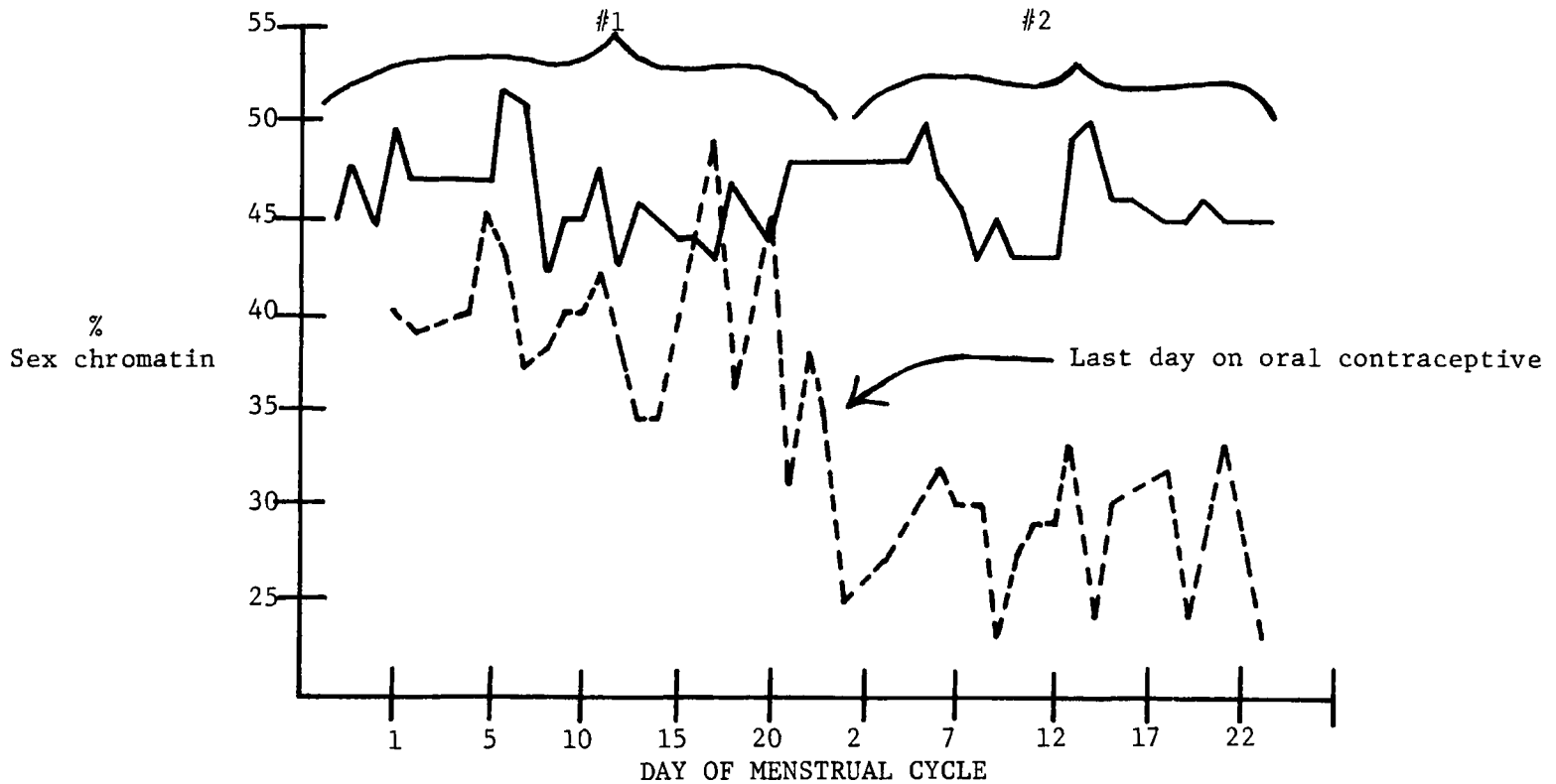


Figure 2. Daily buccal smears of subject III (on Norquen for two menstrual cycles), and subject IV (on Norinyl for first menstrual cycle and off the oral contraceptive for the second menstrual cycle). --Subject III _____ line, subject IV ----- line. Bracket #1 is the first menstrual cycle and bracket #2 is the second menstrual cycle.

Table 2. Statistical Analysis of Subjects on Oral
Contraceptives (Subjects III & IV)

Subject	\bar{X} of menstrual cycle # 1	\bar{X} of menstrual cycle # 2	Comparison between # 1 & 2
III	46.43	46.39	t=0.0624 p > 0.5
IV	39.3	28.1	t=11 p <<< 0.00005

contraceptive (subjects I & II). Women on the birth control pill do not undergo ovulation, but do experience menses. Therefore, the method of analysis for subjects I and II would be unsuitable for subjects III and IV. Subjects III and IV were on the study for a two month period. The data was therefore divided into two menstrual cycles, #1 and #2, for each subject. A comparison was then made between the two menstrual cycles of the subject.

Table 2 shows the statistical data on subjects III and IV. The means of sex chromatin body frequencies for the two menstrual cycles of subject III are almost identical. The difference in the means of the two menstrual cycles for subject IV are statistically significant.

Many investigators have approached the question of whether there is a cyclic fluctuation in the frequency of sex chromatin bodies during the menstrual cycle. There is a great deal of difficulty in correlating all the data from these studies. Every laboratory has its own staining technique and criteria for scoring cells, either sex chromatin plus or negative. The use of different sampling procedures also makes comparisons difficult. Brainerd et al. (1965) took samples on the 1st, 2nd, 10th, 17th, 18th, and 26th days of the menstrual cycle while Dolan (1968) took three samples per period, and Hagy and Brodrick (1972) took daily samples. Even with this great diversity of procedure, most investigators have observed a fluctuation in sex chromatin body frequency during the menstrual cycle.

Subject I's data indicated a mean greater during the ovulatory period than during the periods preceding and following ovulation. The fact that the difference between the ovulatory period and the post and

pre-ovulatory period was not statistically significant could be explained by looking further into the history of the subject.

In 1967 the subject developed an ovarian cyst and subsequently underwent surgery to remove the right ovary. After the operation she went on an oral contraceptive for the next four years. During the period from 1969 to 1970 she became a habitual amphetamine user taking up to 60 mg. a day. Although no work has been done to determine the long term effects of amphetamines and oral contraceptives on sex chromatin, Platt and Kailin (1969) were able to induce a change in the sex chromatin body frequency with bicarbonate.

Subject II on the other hand has no history of ever taking birth control pills or any other types of drugs. She also exhibits an increase in the frequency of sex chromatin during the ovulatory period. In this case, however, the increase is statistically significant.

Figure 3 contains graphs depicting the concentrations of LH, estradiol 17-B and progesterone throughout a 24 day menstrual cycle (Abraham et al. 1971). The graphs show that during the period of ovulation there is a substantial increase in the LH concentration. Just preceding ovulation there is a large increase in estrogen, and following ovulation there is an increase in progesterone. A comparison of the graphs in Figure 1 and Figure 3 reveals a relationship between the sex chromatin body frequency and certain hormones of the menstrual cycle. Section A of subject II, the pre-ovulatory period, has a mean of 32.5; during this period the primary hormone on the rise is estradiol-17 B. The mean of the ovulatory period is 44.6 and corresponds to a high level

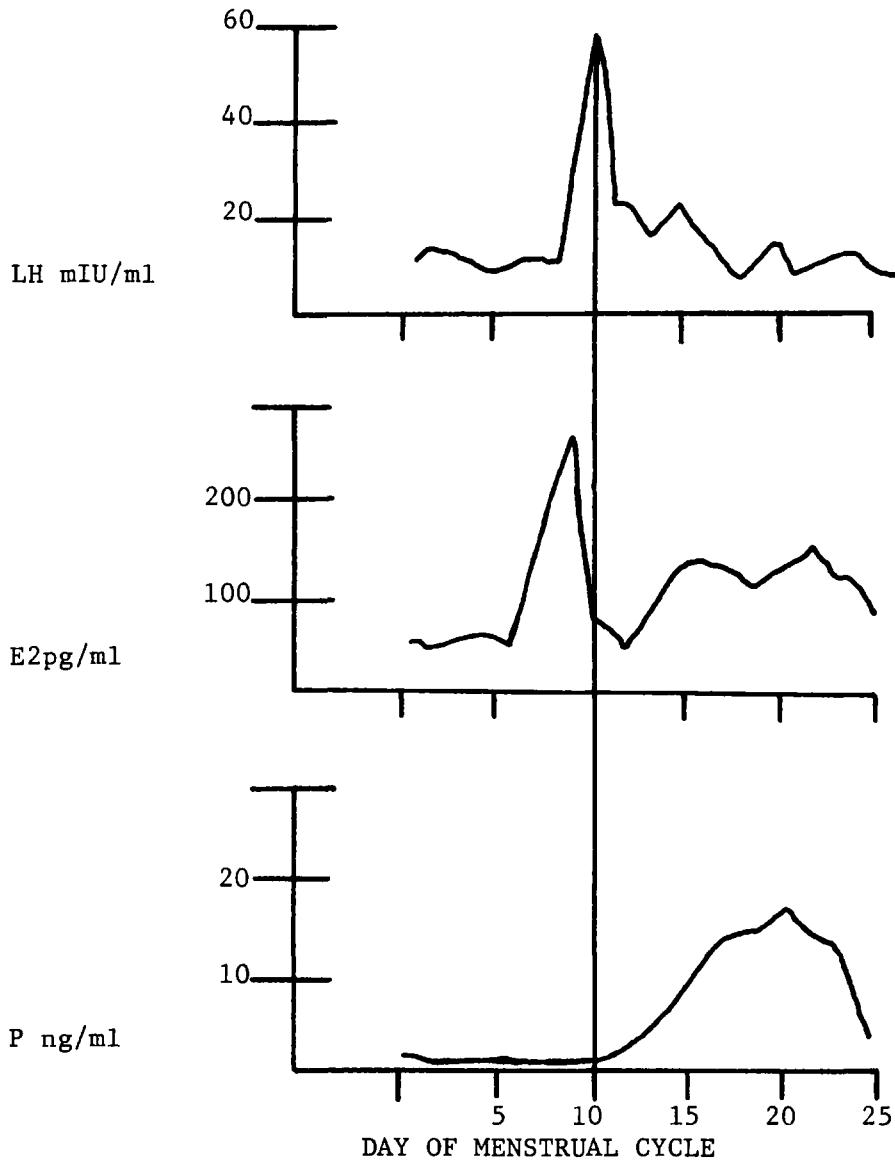


Figure 3. Daily plasma levels of LH, estradiol-17 B and progesterone for women with 24 day menstrual cycles.--(After Abraham et al. 1971).

of estradiol-17 B and an LH surge. The post-ovulatory period has a mean of 29.1 which is slightly lower than the pre-ovulatory period and corresponds to a decrease in LH and estradiol-17 B and an increase in progesterone. There is therefore some relationship between the sex chromatin body frequency and the concentration of these hormones. This relationship is only conjectural in that the hormone levels are a cumulation of a group of women with a 24 day menstrual cycle and not of the subjects tested in this study who had a 22 day menstrual cycle. These results, however, agree with those of Dokumov and Spasov (1967).

The data from Subject IV indicates that the oral contraceptive Norinyl 1+80 28 day has a marked effect on sex chromatin body count. The mestranol is a synthetic estrogen and the norethindrone is a synthetic progestogen which has some progesterone properties and also mimics the estrogens (Appendix A). Women on birth control pills have higher than normal levels of estrogen and lower than normal levels of progesterone. The effect of the high level of estrogen is to act through a feedback mechanism on the pituitary gland shutting off production of LH and therefore stopping ovulation (Dr. Charles Lox, Research Associate, Department of Obstetrics and Gynecology at the University of Arizona Medical School, personal communication, June, 1973).

Subject III maintained a relatively high and steady sex chromatin body count throughout the study. The type of oral contraceptive used by subject III was of the sequential type. The contraceptive properties of this type of pill over the combination pill are greater in that the administration of the mestranol and norethindrone are more closely in tune with the menstrual cycle (Lox, personal communication, June, 1973).

SUMMARY AND CONCLUSION

Daily buccal smears were taken from four volunteer female subjects, one taking an oral contraceptive for the entire study (subject III), one taking an oral contraceptive for the first half of the study and off them for the last half (subject IV), and two who were not on the pill during the study (subjects I & II).

The slides were stained in a 1% solution of cresyl violet acetate then coded by a fellow graduate student and read only by myself. Only nuclei which had smooth oval membranes were counted. A total of 100 cells per slide was read. Graphs were plotted with coordinates % sex chromatin bodies vs. day of the menstrual cycle after all the slides were read.

Daily fluctuation existed in the results of all the subjects tested. An increase occurs in the sex chromatin body frequency during ovulation for the two subjects not taking oral contraceptives. This increase is statistically significant for the one subject who had never taken oral contraceptives and not statistically significant for the subject who had discontinued them two years ago.

The subject on the oral contraceptives for the entire study (subject III), had the most consistent sex chromatin body frequencies. Subject IV, who was on then off the oral contraceptive during the study, showed a significant decrease in sex chromatin body frequency when she discontinued the oral contraceptive.

The data obtained in this study, although from a small test group, lead to several conclusions:

(1) There appears to be a definite cyclic fluctuation of sex chromatin body frequency which corresponds to hormone levels during the cycle.

(2) The oral contraceptives, Norquen and Norinyl 1+80 28 day apparently have an effect on the sex chromatin body frequency. The pills elevate the levels of estrogen in the body, and this elevated estrogen level can be related to the elevated sex chromatin body counts observed in the test subjects.

(3) It would therefore appear that the hormone in the menstrual cycle which is responsible for the elevated sex chromatin frequency is estrogen.

The clinical interpretation of buccal smears should therefore take into consideration whether the subject is on an oral contraceptive, and if not, the day of the menstrual cycle should be determined.

APPENDIX A

STRUCTURES OF HORMONES

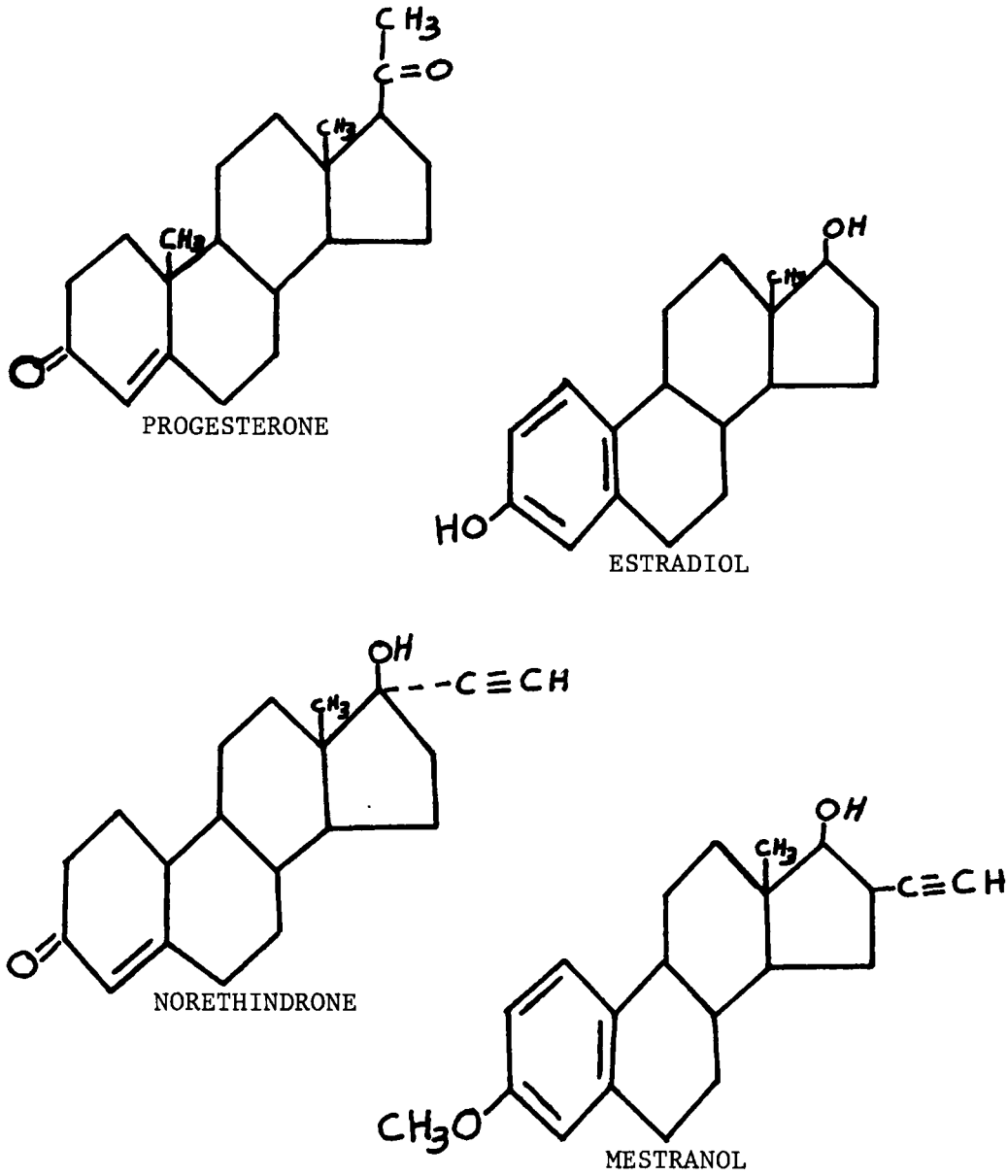


Figure A-1. Structures of hormones.

LITERATURE CITED

- Abraham, G. E., Swerdloff, R. S., Tulchinsky, D., Hopper, K., and Odell, W. D. 1971. Radioimmunoassay of plasma 17-Hydroxyprogesterone: *J. Clin. Endocrin. & Met.* 33: 42-46.
- Barr, M. L. and Bertram, E. G. 1949. A morphological distinction between the neurons of the male and female and the behavior of the nucleolar satellite during accelerated nucleoprotein synthesis: *Nature (London)* 163: 676-677.
- Becker, K. L., Martin, B., and Boukhris, R. 1971. Effect of exogenous estrogen and progesterone upon sex chromatin frequency: *J. Urol.* 109: 79-81.
- Blanco de del Campo, M. S., and Ramirez, O. E. G. 1965. Fluctuations of the sex chromatin during the menstrual cycle: *Acta Cytol.* 9: 251-256.
- Brainerd, T., Mercer, C., and Miles, C. P. 1965. Absence of correlation between sex chromatin incidence and the menstrual cycle: *Acta Cytol.* 9: 440-441.
- Cavelli, I. J., Waldriguez, A., Stueber, N., and Marcallo, F. A. 1970. Sex chromatin and menstrual cycle: *Lancet* 2: 832.
- Comings, D. E. 1967. The duration of the inactive X chromosome in humans based on the persistence of the heterochromatic sex chromatin body during DNA synthesis: *Cytogenetics* 6: 20-37.
- Curtis, D. J. 1969. Sex chromatin frequency in buccal mucosal tissue in the normal female population: *Cytogenetics* 8: 20-29.
- Dokumov, S. I., and Spasov, S. A. 1967. Sex chromatin and sex hormones: *Am. J. Obst. Gyn.* 97: 714-718.
- Dokumov, S. I., and Spasov, S. A. 1968. Sex chromatin pattern and menstrual cycle: *Acta Cytol.* 12: 131-136.
- Dolan, B. E. 1968. Sex chromatin in incidence and the human menstrual cycle: *Acta Cytol.* 12: 128-130.
- Hagy, G. W., and Brodrick, M. D. 1972. Variation of sex chromatin in human oral mucosa during the menstrual cycle: *Acta Cytol.* 16: 314-321.

- Klinger, H. P., Schwerzacher, H. G., and Weiss, J. 1967. DNA content and size of sex chromatin positive nuclei during the cell cycle: *Cytogenetics* 6: 1-19.
- Klinger, H. P., Davis, J., Goldhuber, P., and Ditta, T. 1968. Factors in influencing mammalian X chromosome condensation and sex chromatin formation: *Cytogenetics* 7: 39-57.
- Levij, I. S., and Carel, R. 1968. Variation in incidence of X chromatin: a reappraisal: *Acta Cytol.* 12: 352-356.
- Lyon, M. F. 1966. Sex chromatin and gene action in the X chromosome of mammals, 370-386. *In* The Sex Chromatin, K. L. Moore (ed). W. B. Saunders Co., Philadelphia-London.
- Lysovitch, A., and Margulis, R. R. 1969. Variations in gonadal hormones and the X chromosome: *Am. J. Obst. Gyn.* 105: 269-273.
- McQuarrie, H. G., Scott, D. D., Ellsworth, H. S., Harris, J. W., and Stone, R. A. 1970. Cytogenetic studies on women using oral contraceptives and their progeny: *Am. J. Obst. Gyn.* 108: 659-665.
- Miller, O. J., and Warburton, D. 1968. The sex chromatin I. The significance of distribution of X chromatin counts in individuals with three or more X chromosomes: *Cytogenetics* 7: 58-77.
- Mittwoch, V. 1964. Barr bodies and their relation to nuclear size: *Cytogenetics* 3: 62-74.
- Platt, L. I., and Kailin, E. W. 1969. Buccal X chromatin frequency in numerous diseases. *Acta Cytol.* 13: 700-707.
- Riesenberg, D. W., VanPeenan, H. J., Schmidt, M., Miller, M. V., and Lucas, F. V. 1966. Evidence for activation of the second X chromosomes in females: *Fed. Proc.* 25: 234.
- Riesenberg, D. E., VanPeenan, H. J., Miller, M. V., Schmidt, M. E., and Lucas, F. V. 1967. Further evidence of cyclic activation of the heterochromatic X chromosome in human female: *Am. J. Obst. Gyn.* 97: 91-93.
- Schmidt, M. E., Miller, M. V., VanPeenan, H. J., and Lucas, F. V. 1966. Changes in sex chromatin pattern during the menstrual cycle: *Am. J. Obst. Gyn.* 94: 422-424.
- Seshadri, R., Shan, P. N., and Trivedi, M. B. 1970. Sex chromatin patterns in buccal smears in breast cancer in Indian women: *Acta Cytol.* 14: 3-5.

Stanley, M. A. 1966. Sex chromatin anomalies in female patients with breast carcinoma: *Lancet* 1: 690-691.

Townsend, J. F., Case, M. S., and Lucas, F. V. 1970. Sex chromatin count in pregnancy: *Am. J. Obst. Gyn.* 108: 585-587.

Weste, S. M., Garson, O. M., Barrwett, J. S., and Barkie, A. G. 1967. Variations on the frequency and form of X chromatin in females with burns: *Lancet* 1: 745-746.

