PROPERTIES OF CRYSTALLINE OESTROGENIC HORMONES

THE CHEMICAL AND PHYSIOLOGICAL PROPERTIES OF CRYSTALLINE OESTROGENIC HORMONES.

by

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THESIS

submitted to the Faculty of Graduate Studies and Research in partial fulfilment of the requirements for the Degree of Doctor of Philosophy.

McGill University,
Montreal, Canada.

April 25, 1932.

Acknowledgement.

This investigation has been carried out in the Department of Biochemistry at McGill University under the direction of Dr. J. B. Collip. The author wishes to acknowledge his great debt of gratitude to Dr. Collip for his guidance, advice and encouragement throughout the course of this investigation. Dr. D. L. Thomson's timely suggestions and criticisms have been invaluable to the author during the progress of this work. Acknowledgement is also due to Professor F. E. Lloyd of the Department of Botany for his aid in the micro-photography.

The assistance of Miss J. E. Williamson and Mr. A. Long has been of great help to the writer.

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The Chemical and Physiological Properties of Crystalline Oestrogenic Hormones.

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A crystalline oestrogenic hormone has been isolated from human placenta. This substance shows a melting point of 274°C. (uncorrected). The carbon-hydrogen analysis gives carbon 74.8 per cent, hydrogen 8.8 per cent. The mixed melting point with theelol shows no lowering. The substance differs physiologically from previously isolated female sex hormones. It is relatively inactive on the adult ovariectomized albino rat. The dose required is 16γ as compared to 1.5γ for theelol. It is however, effective on the immature intact rat, 21 days old, in a dose of 1.2γ. This is the same as theelol. On the immature castrate, the dose of the placental crystals is 10γ, that for theelol is 3γ. The presence of the ovary is believed to be the factor in the dosage ratio between adult spayed and normal intact animals. Transplantation of immature ovaries into immature or adult castrates, even though it does not render them cyclic, causes the weight of the placental crystals needed for an effect to fall to the immature intact dose.

Evidence is presented that this substance is the pure form of the hormone emmenin previously prepared in crude form from human placenta by Dr. J. B. Collip. A theory of the mechanism by which the ovary acts on the placental crystals is presented. The importance of the immature rat as a test object for oestrogenic substances is emphasized.

HISTORICAL INTRODUCTION.

The influence of the sex organs and of their removal upon the animal as a whole has been recognized from the very earliest times. The practice of castration and spaying of domestic animals rendered familiar the effects of their removal. The spaying of female animals was not as frequently practised as the removal of the testis because of the relative inaccessibility of the former.

Aristotle in his Historia Animalium remarks, "The fact is that animals if they be subject to modification in minute organs are liable to immense modification in their general configuration. This phenomenon may be observed in the cases of gelded animals; only a minute organ of the animal is mutilated and the creature passes from the male to the female form. We may infer then, that if in the primary conformation of the embryo an infinitesimally small but absolutely essential organ sustains a change of magnitude one way or the other, the animal will in one case turn to male and in the other to female, and also, that if the said organ be obliterated altogether, the animal will be of neither one sex nor the other."

Theories of the Causation of Sex Differentiation.

The mechanism of differentiation which causes a fertilized ovum to develop a testis or an ovary is not clearly understood but is believed to depend upon its chromosome constitution. Once the ovary or testis has been developed, the subsequent differentiation of the soma is believed to be dependent upon stimuli arising from these organs. However, Frank (1929) was unable to show that foetal ovaries possessed any internal secretion. The sex differences between male and female are seen in the presence of an ovary or a testis, in the accessory sex organs and in the secondary sex characters. The secondary sex characters consist of differences in skeleton, in hair distribution, plumage, behaviour, voice, etc.

The relative importance of the influence of the gonad and of the inherited chromosome type present in all somatic cells in the development of sex characters has been well discussed by Riddle (1931). Though the particular combination of genetic factors in the fertilized ovum does determine whether the individual organism shall be male or female, yet a genetic combination which would normally develop into a female can be forced to complete its development as a male, and vice versa. If the primary sex characters are reversed, the accessory and secondary characters are also reversed. The chromosomal difference, though it normally determines it, is not the ultimate cause of sex difference since experimental procedure may secure either sex from the same chromosomal complex. That the chromosomal complex has some influence in the development of sex characters is seen in the case of insects where the control of sex is wholly genetic, the gonads do not influence their

development. Also in certain anomalous cases where gonads were absent in some amphibia and birds, these developed accessory and secondary sex characters of one type or the other. Gynandromorphism or the condition where one part of an organism is male and the other female cannot be explained on hormonal grounds since the internal secretion of the gonad should reach all parts of the body equally. However, it is true Freud, de Jongh and Laqueur (1930) have produced gynandromorphism by simultaneous injection of both male and female hormones.

On the other hand, the possibility of complete reversal of sex before or after development which can be performed in some species (amphibia, birds, mollusca and some worms) is evidence for the great importance of the hormonal influence. Further evidence for this is afforded by the experiments of Steinach (1910) who was able to reverse partially sex characters by removal of the gonad and its replacement by one of the opposite sex.

Riddle (1931) holds that it is possible that the determination of sex depends upon difference of oxidation rate in the early development of the fertilized ovum due to differences in the external environment. He holds that the essential difference between male and female is a difference in oxidation rate of the cells of the soma, the female having the lower rate.

General Description of the Mammalian Ovary.

The ovary is situated in the abdomen attached to the posterior wall by the hilum in which run the blood vessels which

supply it. It is covered by germinal epithelium which is continuous with the peritoneum. In embryonic and pre-pubertial life, the germinal epithelium grows down into the stroma and from these inclusions the ova and probably the surrounding cells of the membrana granulosa later develop. Hargitt (1930) has shown that in the rat new ova are formed in adult life. The germinal epithelium is most active in the non-pregnant animal between oestrous periods. New ova are formed from it during pregnancy and oestrus. In senile animals the germinal epithelium still grows in cords but often no ova are formed and the cords remain as interstitial tissue. The stroma of the ovary consists largely of connective tissue. In some animals, the rabbit for example, there are also present groups of epithelial cells which form the so-called interstitial tissue.

The ovum becomes surrounded by various groups of cells and together with these forms the Graafian follicle. This is made up of two peripheral layers, the theca externa, mostly fibroblastic; and the theca interna which varies in different animals. Within these are the cells derived from the germinal epithelium, the ovum itself surrounded by the polygonal cells of the membrana granulosa. As the follicle develops, in most species a cavity appears in the granulosa cells. This cavity is filled with fluid (the follicular fluid) and gradually enlarges. The number of primary follicles, consisting of the ovum and a few granulosa cells is very large. Only a few of these develop into an actual Graafian follicle. The development of follicles goes on slowly up to the age of puberty, that is, to the time of the appearance of the first oestrous cycle. At this time certain follicles, the number varies in different

species, enlarge rapidly. The cavity becomes much distended with fluid and the follicle protrudes from the surface of the organ. Finally, rupture takes place and the ovum and the follicular fluid escape. This process is known as ovulation. It occurs spontaneously at each oestrous period in most species and recurs at varying intervals throughout the sexually mature life of the individual. The great majority of the Graafian follicles, even those which partially mature, do not proceed to ovulation. They undergo a type of degeneration known as atresia. They may be wholly metamorphosed into connective tissue or they may form atretic corpora lutea.

The normal corpus luteum is formed at the site of the follicle after its rupture. The cavity first becomes filled with blood from the ruptured capillaries of the theca interna. It is later filled with large cells containing the so-called lutein granules. The derivation of the cells of the corpus luteum has been the subject of much controversy. It is now generally believed that they are derived in some species at least from both the theca interna and the remaining cells of the membrana granulosa. In some species,especially the cow, the corpus luteum is hollow and contains some follicular fluid. Allen (1922) for the mouse and Hammond (1927 for the cow, showed that the secretion of follicular fluid goes on temporarily after ovulation and may not be absorbed by the lutein cells but may remain as an occlusion. The corpus luteum after persisting for a varying length of time retrogresses and is either reabsorbed completely or becomes a corpus albicans which consists largely of connective tissue.

The nature and function of the interstitial tissue of the ovary is still in doubt. Clumps of large epithelial cells are very obvious in the ovary of the rabbit, but appear to be absent in the mouse. Some authors apply the term interstitial tissue to the products of follicular degeneration as well as to cells of non-follicular origin. Bouin and Ancel (1909) believe that it occurs only in those animals which do not ovulate spontaneously at oestrus but require the added stimulus of copulation. Lane-Claypon (1907) states that the interstitial tissue undergoes cyclic changes with oestrus, whereas Steinach (1911) regards the interstitial tissue as the chief endocrine tissue of the ovary and has called it the "puberty gland".

General Description of the Mammalian Accessory Sex Organs.

The accessory sex organs in the female are the Fallopian tube, the uterus and vagina, and the mammary glands. They undergo slow development as the young animal grows but remain relatively undeveloped until the onset of puberty when they undergo rapid growth and from that period show, with the ovary, a series of cyclic changes which have been described as the oestrous cycle. The type of oestrous cycle differs greatly in different animals.

The Fallopian tube has a distal expanded funnel-shaped end (the infundibubum) which receives the ovum after ovulation. In some species, as the rat and mouse, the opening is within the ovarian capsule. In others as in man, the cow and the sheep, it is into the peritoneal cavity. The tube may be coiled or straight

and the mucous membrane of the lumen is usually thrown into a series of folds. The epithelium lining the lumen is ciliated and this aids in the passage of the ovum to the uterus. The tube has a circular and a longitudinal muscular coat.

The uterus varies greatly in form in different species. It also has two muscular layers, one circular and one longitudinal, and a layer of mucous membrane. The layers are much thicker than in the Fallopian tube. The mucous membrane of the uterus is known as the endometrium; it contains glands and is lined by epithelium. The shape of the uterus may vary all the way from two distinct horns united even in the cervical canal as in the rat and mouse, to a uterus where the horns are fused up to the beginning of the Fallopian tube, as in the human.

The lower part of the uterus is called the cervix and leads into the vagina which connects the uterine cavity with the exterior. The vagina has two muscular layers and is lined by epithelium which varies in different species and at different stages in the oestrous cycle. In some animals, the vaginal opening to the exterior is closed until puberty. In the embryo the vagina consists of a solid cord of cells which gradually break down to form the lumen, the external cells often persisting up to puberty as mentioned above. In the guinea pig, the membrane closing the vagina is regenerated after each oestrous cycle.

The mammary glands are usually bilateral. They vary in number from one to as many as six or nine pairs according to species. The mechanism of their secretion is not well understood. They differ from other accessory sex organs in being rudimentary when puberty is reached.

Physiological Periodicity in the Female Mammal.

The evidence for the internal secretion of the ovary could not be obtained until the normal changes which occur in the sex organs during the life of the female had been thoroughly established. It is upon this morphological basis that later experimental work rests. The high degree of species difference in these morphological changes made their correlation very difficult.

One may with Parkes (1929) regard the sexual periodicity of the female mammal as consisting of three cycles. The attainment of puberty, sexual maturity, and the decline of sexual function at the menopause is one; the periodic occurrence of the breeding season which appears one or more times a year is the second and the cyclic periods of oestrus at which mating takes place and of which one or more occur during each breeding season, is the third. Allen and Doisy (1927) separate a fourth cycle, the reproductive, which they set aside from the adult non-pregnant period.

In the first period of life, the pre-pubertial, the ovary shows a somewhat irregular development. In the mouse for example, the growth of follicles is quite advanced at three weeks of age but retrogresses again with a decrease in size before puberty which occurs at about six weeks of age. (Brambell 1928). The growth of the accessory organs is gradual and continuous however. Steinach (1911) regards this development as occurring under the influence of the interstitial cells of the ovary.

At puberty, there is an abrupt appearance of an oestrous cycle. This first cycle differs in no way from other cycles which

follow. The age at puberty differs according to species and will be discussed below in connection with the various types of oestrous cycle.

The fading out of the sexual cycle is much less abrupt than its onset. In the human it may take two or three years. The cessation of the sexual cycle in the lower mammals has not often been observed because in the ordinary course of events, they either die or are killed before it occurs. In the laboratory, the increased susceptibility to disease usually results in death before or at the time when sexual function declines. Parkes (1929) gives the following stages for the mouse:-

(1) Litter size gradually decreases.

(2) Coitus becomes infertile owing to ovulation having ceased.

(3) Oestrous symptoms in the accessory organs become infrequent and irregular.

(4) Complete anoestrus appears.

In the last stage, the ovary is devoid of follicles and of corpora lutea and the accessory sex organs have retrogressed and show fibrotic changes.

The Sexually Mature Period.

In many domesticated animals and also in man, oestrus occurs at regular intervals throughout the entire year. A constant food supply and existence under equable conditions of temperature probably have something to do with this regularity. In the wild state, on the contrary, animals show a definite period of the year at which mating occurs--this usually falling at such a time that,

according to the period of gestation, the young will be born at a suitable time of year as far as food and temperature conditions are concerned.

In each breeding season mating occurs only at certain definite periods known as oestrus or "heat". There may be one or more of these in each breeding season. The occurrence of pregnancy causes a cessation of the oestrous cycles. During the non-breeding part of the year the reproductive organs are in a state of rest--the anoestrous period. If the gestational period is very long (as in mares, 11 months) the anoestrous period is occupied by pregnancy. Even in domesticated animals there is usually a time of year when breeding occurs readily. This probably corresponds to the original breeding season of the species. The cause of the onset of the breeding season may be related to increased environmental temperature and food supply. Lee (1926) found an increase in length of oestrous cycle of 80 per cent over a period of three months when he exposed rats to an environmental temperature of 2-10°C. Parkes (1928) found a similar increase in the mouse but this increase was not maintained. He regards the low food supply as the cause of anoestrus in the wild rodent during the winter months. It is well known that poor nutrition causes prolonged and irregular cycles in laboratory animals.

The Main Characteristics of the Oestrous Cycle.

The ovarian cycle has been described above. It consists of maturation of follicles, discharge of the ova and formation of corpora lutea with subsequent degeneration and fresh follicular maturation. With these changes may be correlated those occuring

in the accessory organs.

Oestrus is the point in the cycle at which ovulation and copulation take place. In most animals it is at this period only that the female will accept the male. Before oestrus proper there is a preliminary period called pro-oestrus during which the follicles in the ovary increase in size and some growth takes place in the accessory organs. Oestrus is usually followed by a period of further development in ovary and accessory organs called metoestrus. What happens after this depends on the species of animal, the fate of the ova, and the growth of the corpus luteum. In the absence of pregnancy and in animals which have only one oestrous period during the breeding season, a return to the an-oestrous state takes place at once. If, however, the animal is polyoestrous, it shows a short intervening period called the di-oestrous interval. At the end of this period pro-oestrus again occurs and the cycle repeats itself. The essential feature of this type of cycle is the presence in the ovary at the same time of both growing follicles and of corpora lutea. The length of the dioestrous interval is controlled by the time of persistence of the corpus luteum. If pregnancy occurs, it persists throughout the period of gestation. In the absence of pregnancy in the rat and mouse its development is very transient normally. In Marsupials and in the ferret it may undergo development equal to that of pregnancy and then striking growth effects occur in the accessory organs. This condition is known as pseudo-pregnancy. It does not occur in all species. Ovulation and corpus luteum formation are

essential for its development. In the rabbit and ferret, however, ovulation only occurs after copulation and these animals are in a state of continuous oestrus throughout the breeding season and at the end of this time, if copulation has not taken place, follicular atresia takes place and with it the onset of the anoestrous period. Pseudo-pregnancy may be induced in the rat and mouse by sterile copulation. Its duration (12 days) is about half that of normal pregnancy.

The participation of the accessory sex organs in the cyclic changes of oestrus is not the same in all species. In all, the uterus shows changes. In the ferret, Marshall (1904), guinea-pig, Stockard and Papanicolaou (1917), rat, Long and Evans (1922), and mouse, Allen (1922), well-marked vaginal changes also occur. The mammary glands are involved in cyclic changes during oestrus in some species. There are also changes in secondary sex characters with breeding season such as alterations of plumage in birds, etc. but these are not striking in mammals where the great changes in secondary sex characters (voice, hair distribution, etc.) occur at puberty. There are, however, alterations in behaviour and the appearance of certain special reflexes at the time of oestrus.

A summary taken from Parkes (1929) showing the terms used with the corresponding phases of the oestrous cycle in the sex organs is presented below in table I.

TABLE I.

PHASE	OVARIAN CYCLE	UTERINE CYCLE
Anoestrus	Rest.	Rest.
Prooestrus	Maturation of follicles.	Growth.
Oestrus	Ovulation.	Degeneration.
Metoestrus	Formation of corpus luteum.	Recuperation.
Dioestrus	Transitory development of corpus luteum.	Transitory or no development.
Prooestrus	Maturation of follicles.	Growth.
Pseudo-pregnancy	Development of corpus luteum.	Extensive development.
Anoestrus	Rest.	Rest.

The Types of Oestrous Cycle in Various Species.

The accompanying table (table II) is a comparison of some of the cyclic phenomena in a number of species.

TABLE II.

Comparative Physiology of the Oestrous Cycle.

Species Type of Cycle	Phase	Duration (Days)	Ovary	Uterus	Vagina	Mammary Gland	Age at 1st Oestrus
Ref.: (Hill and O'Donoghue, 1913)							
Dasyurus viverrinus: Monoestrous type.	1.Prooestrus	4-12		Mucosa thick and vascular. Glands lengthen and become convoluted. Epithelium thickens.	-	No growth.	-
	2.Oestrus	1-2		Same condition.	-	No growth.	-
	3.Post oestrus	5-6	Ovulation spontaneous at end.	Some regression.	-	No growth.	-
	4.Pseudo-pregnancy	8-14	Corpus luteum develops.	Further growth of mucosa.	-	Growth.	
	5.Pregnancy						
Ref.: (Marshall and Jolly, 1906; Gerlinger, 1923; Evans and Cole,1931)							
Dog: Monoestrous type.	1.Anoestrus	-	Small. Growth begins one month before oestrus.	Thin, anaemic mucosa few glands. Growth begins in anoestrus.			
	2.Prooestrus	-	Follicular maturation.	Thickens. Capillaries increase. Secretion from glands.	No change.	No growth.	
	3.Oestrus	-	Ovulation spontaneous. Corpus luteum degenerates from 20th day.	Capillaries and epithelium break down. Blood extravasated into lumen.	Blood appears externally.	No growth.	10 mos.
	4.Pseudo-pregnancy	60	Corpora lutea well developed. Degenerate at end.	Mucosa greatly thickened. Glands numerous and coiled. At end extravasation of blood in stroma but epithelium differs from prooestrus.	Blood appears externally at end.	Growth. Breaks down. Milk secreted. at end.	
	5,Pregnancy	60	Corpus luteum persists throughout.	Greater growth than (4). (Oestrus absent during lactation)	-	Growth greater than (4)	

TABLE II.

Comparative Physiology of the Oestrous Cycle. (Continued)

Species Type of Cycle	Phase	Duration (Days)	Ovary	Uterus	Vagina	Mammary Gland	Age at 1st Oestrous
Ref.: (Loeb,1911,1914) (Stockard and Papanicolaou, 1917)							
Guinea-pig: Polyoestrous type	1.Oestrus Stage I.	6-12 hrs.	Mature follicles.	Mucosa thickens. Glands increase.	Membrane breaks down. Will accept male. Contents: mucous secretion cornified cells. Mucosa: epithelium being shed, infiltration of leucocytes beginning.	Growth	-
	Stage II.	2-4 hrs.	Ovulation.		Contents: cheesy, great numbers of nucleated epithelial cells. Mucosa: leucocytes increasing.		
	Stage III.	4-6 hrs.			Contents: fluid thinner. Leucocytes appear. Mucosa: congestion. Large numbers of leucocytes. Some hemorrhage, then regeneration.		
	2.Dioestrus	16	Corpus luteum fully formed at 5th day post oestrus. Degenerates 10th day. Increased follicular growth for 8 days. Then atresia. Second increased growth culminates in oestrus.	Regression begins 4th day post ovulation. Growth begins 12th day.	Closed by membrane renewed each cycle.	Regression.	
	3.Pseudo-pregnancy		Corresponds to dioestrous period.				
	4.Pregnancy	62	Corpus luteum persists. Degenerates on 40th day. Waves of follicular growth throughout every 8 days followed by atresia.		Contents: leucocytes.	Growth.	

TABLE II.

Comparative Physiology of the Oestrous Cycle (Continued)

Type of Cycle	Phase	Duration (Days)	Ovary	Uterus	Vagina	Mammary Gland	Age at 1st Oestrus
Ref.: (Hammond, 1927)							
Cow: Polyoestrous type	1.Oestrus	12-24 hrs.	Ovulation spontaneous 0-24 hours post oestrus.	Large amount of fluid secreted.	Some hemorrhage from vaginal stroma immediately post oestrus.	No definite growth	15 mos
	2.Dioestrus	20 hrs.	One large follicle 8 days post oestrus. Corpus luteum fully formed 8 days post oestrus.	Congested for 8 days post oestrus.	Mucosa develops for 8 days post oestrus. Then desquamation.	Regression in second half.	
	3.Pregnancy	240 hrs.	Corpus luteum enlarges.	Great enlargement. (Oestrus occurs normally during lactation)		Growth.	
Ref.: (Hammond, 1925)							
Rabbit: Persistent oestrus type	1.Oestrus	-	Follicles mature and persist till copulation or end of breeding season.	Growth and vascularization at beginning of breeding season.		Ducts grow out at first oestrous period. Then no change.	6 mos
	2.Pseudo-pregnancy	14	Ovulation 10 hours post coitus. Corpus luteum for 14 days.	Immense growth of glands and stroma with secretion of fluid.	No definite changes.	Further growth laterally. Alveoli develop. Degenerate at end. Small amount of milk.	
	3.Pregnancy	30	Ovulation 10 hours post coitus. Corpus luteum persists.	Similar to (2) but more marked. (Oestrus occurs during lactation but pregnancy does not)		Same as (2) for 14 days, then growth in depth as well.	

TABLE II.

Comparative Physiology of the Oestrous Cycle. (Continued)

Species Type of Cycle	Phase	Duration (Days)	Ovary	Uterus	Vagina	Mammary Gland	Age at 1st Oestrus
Ref.: (Horner, 1921)							
Pig: Polyoestrous type	1.Oestrus	3	Ovulation towards end.	Epithelium low columnar closely packed. Glands not enlarged. Stroma oedematous. Many leucocytes.	Vulva swollen.		6 mos.
	2.Dioestrus	18	Corpus luteum maximum 8 days post oestrus. Then regresses. New follicles grow 3 days before oestrus.	Further growth in mucosa for 7 days. Epithelium columnar glands proliferate. At 10th day epithelium reduced to low columnar.			
	3.Pregnancy	120	Corpus luteum persists.	Ova implanted 8-10 days after ovulation. Further development of stroma. (Oestrus occurs normally during lactation)			

The Menstrual Cycle in Primates.

The oestrous cycle in primates in contrast to all the above species, shows the phenomenon of menstruation. The significance of this and its relation to the oestrous cycle of lower mammals is not yet clear. Heape (1894) observed in Semnopithecus entellus that a regular menstrual cycle could occur in the absence of ovulation and in the absence of a corpus luteum. The reverse condition that is ovulation and fertility with complete absence of menstruation,has been observed in some human cases. Frank (1929).

The uterine cycle is similar in monkeys and in the human. It has been divided into four stages: (1) Period of Rest (2) Period of Growth (3) Period of Destruction and (4) Period of Regeneration. Williams (1922) gives for the human a duration of 12 days for the first period, five for the second, four for the third, and seven for the last. During the period of growth the uterine mucosa becomes greatly thickened; there is a superficial compact layer beneath the surface epithelium and below this the gland alveoli are distended and the stroma is oedematous. Beneath this layer is a third, the basal layer where there is no oedema or hypertrophy. These changes increase until the 28th day when disintegration sets in, in the absence of pregnancy. There is a break down of the superficial and middle layers of the mucosa and extravasation of blood occurs under the compact layer. This blood finally reaches the lumen and appears as the menstrual flow. Regeneration occurs from the basal layer.

No definite vaginal cycle occurs in the primate, though

there are slight changes in the proportion of leucocytes present in the vaginal contents. Mammary hypertrophy occurs in the human in the immediate pre-menstrual phase but is absent in most species of monkey.

The pre-menstrual growth of the uterus has been regarded as analogous to the pseudo-pregnant condition of the rabbit and other species and as dependent upon the corpus luteum. Cannon (1931) found that removal of the corpus luteum in the human did not hasten the next menstrual period. The possibility of menstruation without ovulation and corpus luteum formation as mentioned above, indicates that the process is not wholly dependent upon the corpus luteum. The pre-menstrual growth does however occur at a time when the corpus luteum is developing. A second theory regarding menstruation is that it corresponds to the prooestrous haemorrhage occurring in the dog and other species (see table II). This belief is strengthened by the production of menstruation at the cessation of a series of injections of oestrus-producing hormone into the Macacus monkey by Allen (1927). He also produced it by rupturing ripe follicles in the ovary. A point against this view is the wide interval (14 days) between ovulation which occurs at oestrus in lower animals, and menstruation. The view that ovulation takes place only mid-way in the cycle of menstruation is that of Schroeder (1928). Evans and Swezy (1931) from their own and other data consider that ovulation may occur at any time in the menstrual cycle. They also think that bleeding may take place without any great destruction of uterine epithelium and that it is not always that a great increase in growth of the endometrium with subsequent breakdown occurs. They would

regard the cycle of bleeding and the utero-ovarian cycle of oestrus as not necessarily connected. Marshall (1927) has recently suggested that menstruation represents both pseudo-pregnancy and prooestrus telescoped together, since in many mammals prooestrus immediately succeeds pseudo-pregnancy and if the cycles were contracted at that point, they might overlap. This would explain the occurrence of menstruation without ovulation as consisting of the prooestrus part only. In this connection Corner (1927) has observed that in the case of menstruation without ovulation in monkeys, the typical pre-menstrual growth of the endometrium does not occur and no desquamation occurs at menstruation. Cannon (1931) suscribes to the double origin theory of menstruation with slight modifications as to the degree of overlapping of the two phases.

The Oestrous Cycle in the Rat and Mouse.

The rat and the mouse have very similar oestrous cycles. The rat was the animal used in the investigations undertaken in the preparation of this thesis. Prior to the discovery of the marked vaginal cycle in these animals, the length of cycle was put at varying figures from ten to twenty days. Long and Evans (1922) worked out the vaginal cycle first observed in the guinea-pig by Stockard and Papanicolaou (1917). Allen (1922) did the same for the mouse. The average length of cycle for the rat was 5.4 days though 82 per cent of the animals showed an average of 4.6 days. In the mouse the length of cycle is 5-6 days. Long and Evans found the first occurrence of oestrus at about the age of 72 days. The

vagina usually opened a few days before the first ovulation. The first cycles tend to be rather longer than the normal for the adult. The age at first oestrus varies considerably with different colonies, being considerably earlier than this in many cases. Curtis and Doisy (1931) found vaginal opening as early as 35-39 days of age. The average in the colony in Dr. Collip's laboratory was 64 days with the earliest observed at about 43 days (C.W.Chapman: personal communication). In the mouse the age at first oestrus is about 7 weeks. Pseudo-pregnancy occurs after sterile copulation or stimulation of the cervical canal, and lasts for about 13 days in both species. The next oestrous cycle is delayed until the end of this time. Since the acceptance of the female by the male was the criterion of oestrus in earlier work, this explains the longer cycles given for these species before the use of the vaginal smear. Oestrus occurs 24 hours after parturition and then ceases for the rest of lactation, about 3 months in the mouse, and 25-40 days in the rat. If pregnancy occurs at the first post-partum oestrus, lactation is not inhibited. The implantation of the embryos may, however, be delayed and pregnancy be prolonged.

The Ovarian Cycle.

In these species, the corpus luteum persists histologically long after it has ceased to function; four or five sets may be present in the ovary at the same time. A number of follicles mature rapidly just before oestrus. In the rat ovulation occurs about 18 hours after the beginning of vaginal cornification.

During oestrus the cavity formed by the peri-ovarian membrane becomes filled with fluid. This facilitates the passage of the ova into the Fallopian tube which opens into this cavity.

The Uterine and Vaginal Cycle.

These are very well marked and are shown in table III, taken from Long and Evans (1922). The vaginal changes are especially important because of their use as a means of detecting the occurrence of oestrus in the living animal.

The leucocytes in the vaginal smear were found during metoestrus in the rat by Post (1927) to be mainly (98-100%) polymorphonuclear in type. A few lymphocytes and monocytes also occurred. During pregnancy the vaginal smear shows a few nucleated epithelial cells and leucocytes. Much mucus is present and on the 12th day blood appears, due to uterine haemorrhage. In pseudo-pregnancy a similar smear is seen and 3 days before the next oestrus, a small amount of blood appears.

The mammary glands show development with each oestrus period and slight regressive changes during dioestrus. They develop to a greater extent during pseudo-pregnancy.

The Role of the Ovary as an Endocrine Organ.

As may be seen from the above summary of the cyclic phenomena, the changes in the accessory sex organs are closely synchronized with those in the ovary. This suggests that the ovary is the organ concerned in their production. The mechanism by which the gonad

TABLE III.
(From Long and Evans)

Summary of Oestrous Cycle Changes in the Rat.

Stage	Living Animal	Histology of Vaginal Mucosa	Uterus	Ovary and Oviduct
1 (12 hrs.)	Vaginal mucosa slightly dry. Smear of epithelial cells only. Lips a little swollen. In heat towards end.	Many layered (8-12) .08-.1 mm. Mitoses active. Cornified layer under superficial layer of epithelium. No leucocytes.	During Stage 1, uterus becomes distended with fluid increasing in diameter from 2.3-3.7 mm.	Follicles large.
2	Vaginal mucosa dry and lustreless. Smear of cornified cells only. Lips swollen. In heat.	7-11 layers of cells. .08-.1 mm. thick. Cornified layer well formed and superficial. No leucocytes. Mitoses fewer.	Reaches greatest distention (5 mm.) and thinness of epithelium and then retrogresses to diameter of 1.8 mm. Vacuolar degeneration sometimes begins.	Follicles largest. Eggs may undergo maturation.
3 (2 and 3)	As in Stage 2, but cornified material abundant (cheesy) and animal not in heat.	5-9 cells thick. .064 mm. thick. Cornified layer loose and finally completely detached. No leucocytes. Mitoses still fewer.	Diameter of uterus about 2.0 mm. Epithelium undergoing vacuolar degeneration.	Ovulation. Secretion of fluid into periovarial space and oviduct.
4 (6 hrs.)	Vaginal mucosa slightly moist. Smear of cornified cells and leucocytes. Swelling of lips gone.	4-8 cells thick. .062 mm. thick. Cornified layer gone. Many leucocytes. Mitoses increasing.	Diameter of uterus 2.2 mm. Some vacuolar degeneration, but also regeneration.	Young corpora lutea. Eggs in oviduct. Follicle smallest.
5 Dioestrous interval (57 hrs.)	Vaginal mucosa moist, glistening. Smear of leucocytes and epithelial cells. Variable amount of mucus.	4-7 cells thick. .042 mm. thick. Leucocytes. Mitoses not numerous.	Diameter 1.7 mm. Epithelium undergoing regeneration.	Follicles of various sizes. Corpora lutea continue to grow. Eggs traversing oviduct throughout early interval.

performs this function has been shown to be of the nature of an internal secretion. The nervous system is believed to play a minor part in the interrelations of ovary and accessory organs. The reflex production of ovulation in the rabbit by stimulation of the cervix shows, however, that the nervous system does play some role. Also,the nervous system as a whole takes part in the behaviour changes and reflexes which accompany oestrus.

The Theory of Internal Secretions.

The conception of the presence of internal secretions,- that is, the secretion of substances by the organs of the body into the blood stream, was first suggested by Théophile de Bordeu (1722-76), a physician at the court of Louis XV. He believed"that not only each gland but each organ in the body is the workshop of some specific substances or secretion which passes into the blood, and that upon these secretions the physiological integration of the body as a whole depends." (Garrison, 1922). Claude Bernard in 1857 obtained glycogen in the pure state and said of the liver, "It seems to unite in effect two distinct secretory elements, and it represents two secretions, one external, the biliary secretion; the other internal, the glycogenic secretion which is discharged into the blood". In 1893, Brown-Sequard stated, "We admit that each tissue and more generally each cell of the organism secretes on its own account certain products or special ferments which through this medium (the blood) influence all other cells of the body; a definite solidarity being thus established among all the cells through a mechanism other than the nervous system-----the tissues (glands or

other organs) have thus a special internal secretion and so give the blood something more than the waste products of metabolism. The internal secretions, whether by direct favorable influence or whether through the hindrance of deleterious processes, seem to be of great utility in maintaining the organism in its normal state."

The conception of the body cells as a whole, secreting substances of this type, is still tenable---for example, one may consider the regulation of respiration by carbon dioxide to place it in this class. The idea of internal secretion has, however, come to be limited to certain special organs which have as their function, partly or wholly, the elaboration of regulatory substances. The term "hormone" for secretions of this type was first used by Bayliss and Starling (1902) in connection with their discovery of secretin. These hormones are regarded as chemical substances which pass into the blood stream and produce their effects on other parts of the body; their action may, however, be limited to one particular organ. In the case of secretin, this organ is the pancreas.

The proof that an organ possesses an internal secretion depends upon the occurrence of certain changes in the organism following upon the removal of the organ. These changes are prevented or reversed by the transplantation of the organ to another part of the organism where it establishes vascular connections but no longer has its normal nerve supply. This nerve connection is thus shown to be unnecessary for the effects of the organ on the body. The changes and their reversal by transplantation indicate that the organ must have been producing some substance while it

was in the body in the normal state. Finally, the preparation of an extract from the organ which will reverse the effects of its removal and the purification of that extract to the pure chemical substance or substances responsible for the effect, establishes firmly the presence of an internal secretion. One should, however, note that the preparation of a chemical substance having certain effects when injected into the organism is no criterion that the substance in question ever existed in this form in the organism, or if it did exist, that it was ever liberated from the organ into the blood while in the body. The posterior pituitary body is a good example of an organ from which a large number of very active principles may be prepared, none of which have as yet been proved to be liberated from the gland while it is in the body.

The Evidence for an Internal Secretion of the Ovary.

In the case of the ovary, the evidence for its internal secretory activity rests upon the effects of its removal (ovariectomy) and of transplantation experiments; also, upon the effects of injection of extracts prepared from it, and recently upon the isolation of chemical substances capable of producing characteristic effects upon the accessory sex organs. Once having established that the ovary is an internal secretory organ, it is necessary to indicate what part of the organ exerts this function, and whether any other organ of the body is capable of producing similar effects. The presence of more than one hormone in the ovary perhaps exerting different effects, must also be considered. The mechanism by which the ovary produces its internal secretion and the stimulus for its

production, whether within the organ itself or external to it, is also essential for a complete understanding of the subject.

The Effects of Ovariectomy.

In the bird, the presence of the rudimentary right gonad which may develop into either testis or ovary on removal of the functional gland, makes possible the reversal of sex by ovariectomy. In the castrated hen, there is an appearance of a neutral capon type; spurs and feathers take on male characteristics but wattles and comb show little change.

In the female mammal, complete gonadectomy does not lead to the assumption of male secondary characters. The female secondary characters are not as obvious as those of the male and so do not undergo such marked changes. The effect is more marked if the animal used is an immature one. Some of the effects observed in the ovariectomized female are the following (Parkes, 1929):

1. The long bones undergo increased growth due to non-union of epiphyses.

2. Greater weight is attained.

3. Sexual instinct is repressed. Any special reflexes as the "tail erection" reflex and the "kick-guarding" reflex in female rodents disappear.

4. Most observers report a slight lowering of metabolic rate following ovariectomy. Loewy and Richter (1889) were the first to observe this. The degree of lowering has been much disputed. It may be partly accounted for on the basis of increased amount of non-metabolizing tissue (fat). Ptaszek (1929) using dogs obtained

marked falls of the order of 40-50 per cent about two or three months after operation. This seems to be rather large. Some authors feel that this decrease is only an occasional phenomenon. (Grafe, 1923). Lee and Van Buskirk (1928) report a slight decrease of about 10 per cent in a small series of male and female rats. There is a variation in the spontaneous activity of the female rat with the oestrous cycle (Slonaker, 1924). This activity is greatly decreased after ovariectomy and so does not run parallel to the change in metabolic rate which is only slight. Also the peak of metabolic increase with oestrus does not take place at the same time in the cycle as the time of greatest activity (Lee, 1928).

The effect on the other internal secretory organs or removal of the ovary has been frequently studied with varying results. There is a definite enlargement of the anterior pituitary gland (Hammond, 1913). The so-called "castration cells" of an eosinophile nature appear in the pituitary after ovariectomy.

The Effect of Ovariectomy upon the Accessory Sex Organs.

Ovariectomy causes great changes in the accessory sex organs and an immediate cessation of all cyclic phenomena. The effect is especially marked if the operation is performed before puberty. If the ovary is removed in adult life, the accessory organs gradually degenerate in a rather similar way to the degeneration which occurs normally at the menopause. This degeneration has been studied in the rat by Marshall and Jolly (1907). The uterus shows degeneration in both muscular and mucosal layers. The glands

undergo atrophy. The changes are complete in about 3 to 4 months. If the operation is performed before puberty, the uterus undergoes regression even from its undeveloped state. This is especially marked in the mouse (Parkes and Brambell, 1927). The vagina also fails to develop and degenerates.

The effects of removal of the ovary were attributed to disturbance of vascular supply of the uterus, by Hofmeier (1880) and to disturbance of the nerve supply, by Sokoloff (1896). Careful precautions against these disturbances and more especially, the reversal of the effects by the transplantation of the ovary, have disproved these theories. The idea that removal of the uterus caused degeneration of the ovary has also been disproved.

The Effects of Ovarian Transplantation.

The reversal of effects of ovariectomy by the transplantation of the ovary into an abnormal site, definitely disposed of the idea that the effects of removal of the ovary were due to the disturbance of nervous connections. Knauer (1900) and Halban (1900) made the first systematic investigation of the transplantation of ovaries into mammals. Knauer used rabbits and employed both auto-transplantation (into the same individual) and homo-transplantation (into an individual of the same species). It should be noted that Marshall (1922) uses the term homo-transplantation in the sense of auto-transplantation and hetero-transplantation in the sense of homo-transplantation used above. Knauer found that part of the graft degenerated but the rest persisted and formed fertilizable

ova and that no castration effects appeared in the animal. Halban used young guinea-pigs and employed auto-transplantation. The guinea-pigs developed normally. Marshall and Jolly (1907, 1908) used the rat as an experimental animal. They used both auto- and homo-transplantation. The graft persisted rather longer in the first case than in the second. The sites for transplantation most commonly employed were the peritoneum, muscle and kidney. The spleen has also been used by later workers. Marshall and Jolly found that the follicles of transplanted ovaries tended to undergo atresia and the corpora lutea to degenerate. Ribbert (1898) found that the central part of the graft first showed the formation of connective tissue and later developed follicles again, presumably with the establishment of an improved blood supply. Marshall and Jolly found that the graft grew in size if a satisfactory blood supply had been established. Auto-grafts were normal 14 months and homo-grafts 6 months after the operation. They frequently showed, however, signs of follicle atresia and of atretic corpora lutea. The graft may come to consist wholly of lutein tissue. Transplantation also leads to the development of large amounts of interstitial tissue in species where it originates from atretic follicles. Cystic follicles may also occur in the transplanted gland. The uterus was prevented from showing any castration atrophy by the graft. If atrophy had already taken place due to previous castration, the condition of the organ was restored to normal. This reversal of effect was dependent upon the survival of the graft. If the latter degenerated, the uterus again showed retro-

gression. A normal sex cycle with normal behaviour at oestrus was maintained in the transplanted animals as long as the graft survived normally. The possibility of pregnancy occurring from the fertilization of ova from transplanted gland has been frequently shown in a number of different animals. It has not been noted whether the ovarian graft will maintain the oestrous cycle after the atresia of the follicles, but Parkes (1929) states that such a graft can maintain the normal nutritional condition of the uterus. Pincus (1930) claims to have transplanted mouse ovaries into rats producing normal oestrous cycles and to have succeeded in getting a litter from one of these animals. The progeny were rats, not rat-mouse hybrids. Steinach (1913) and Lipschutz (1917) as well as many others have investigated the effects of ovarian transplantation into male animals. The grafts do not often develop corpora lutea, in contrast to those in female animals. They tend to degenerate rather earlier. In castrated males they cause partial reversal of sex especially if they are transplanted before puberty. Fea (1900) showed that immature ovaries transplanted into adult female castrated rats showed great rapidity of development and caused oestrus within a few days of their transplantation.

Parabiosis experiments by Martens (1929) and others have shown that a castrate rat when united to a normal rat so that there is a vascular connection between them, is brought into oestrus. This is a proof of the passage of the hormone through the blood stream of the normal rat and into that of the ovariectomized one.

Implantation, that is the introduction of a macerated

tissue intramuscularly where it is absorbed, as opposed to transplantation where the tissue forms a vascular connection, has been used for the ovary by Zondek and Ascheim (1925) with positive effects on castrate animals.

The Use of Ovarian Extracts.

Early Preparations and Criteria of Activity.

One of the earliest attempts to reverse the effects of ovariectomy by the use of ovarian preparations, was that of Landau (1896) who fed dried gland by mouth to humans with very poor results. Jentzer and Beuttner (1900) and Bucura (1907) prepared saline extracts which were ineffective. The criterion of activity in most of the early work was the prevention of castration atrophy in the uterus. The rabbit was the animal most used. Marshall and Jolly (1906) produced transient oestrus changes in the anoestrous bitch by the use of saline extracts. Hermann (1915) and Fellner (1913) used swelling and hyperaemia of external genitalia which occur in rabbits, pigs and dogs during oestrus. They also used the effect on the uterus and that on the mammary gland. Fellner was the first to use the castrate animal systematically as a test object, and to make careful comparison of the results. When the cyclic nature of the changes in the uterus were appreciated, the comparison of effects produced by extracts on the castrate uterus were compared with the normal appearance of this organ in oestrus and even in pregnancy. The results seem to show that the appearance of oestrus was sometimes induced but not that of pseudo-pregnancy.

Adler (1912) claimed to have produced oestrus changes in virgin rabbits by aqueous extracts of whole ovary. Halban (1905) was the first to hold that placenta contained a substance capable of producing oestrus. Sonnenberg (1907) failed to cause oestrus by the injection of follicular fluid extract. He, however, only used very minute quantities and observed the animal for only 30 minutes after the injection; much too short a time for any effect to appear.

A considerable advance was made by Iscovesco (1912, 1913) who showed that the active fraction was soluble in lipoid solvents, and could thus be separated from the proteins. He was able to cause rapid hypertrophy in the uterus of adult rabbits. Fellner (1912, 1913) analyzed the effects of extracts of various reproductive organs upon normal and ovariectomized animals. Extracts containing activity were prepared from placentae, ovaries without corpora lutea and Graafian follicles. He found the principle to be soluble in alcohol, ether and acetone, and concluded that it was of a lipoid nature. Okinschitz (1914) showed that subcutaneous injection of ovarian extracts retarded the atrophy of the uterus in ovariectomized rabbits. Extracts of corpora lutea did not have this effect. This negative result is important since at that time the theory of Fraenkel (1903) that the corpus luteum was the only part of the ovary which produced an internal secretion, was widely held. Hermann (1921) used immature rabbits and emphasized the presence of the oestrus-producing substance in placenta. Frank (1922) obtained a positive result (hypertrophy of the uterus in normal rabbits) by the injection of untreated follicular fluid.

The fluid from the ovaries of non-pregnant cows was more effective than that from those of pregnant ones.

In 1923 a great advance was made with the introduction by Allen and Doisy of the vaginal smear technique for the detection of the active substance. This was based upon the cyclic changes present in the vagina of the female rodent which were described by Long and Evans (1922) after they had been discovered in the guinea-pig by Stockard and Papanicolaou (1917), and have been summarized above. The appearance in the castrated rat of the reaction characteristic of oestrus (that is, a smear consisting wholly of cornified cells) was their criterion of activity, the unit being defined as the amount of substance just sufficient to cause full cornification in the vagina of an adult ovariectomized rat. The technique of taking the smear of the vaginal contents will be discussed under the section on assay. The advantages of this method were the possibility of using the living animal for observation. The normal conditions had been well established and a smaller amount of material was needed, thus conserving material.

The Distribution of the Active Substance.

Before continuing with the advances in preparation and physiology, a discussion of the sources and distribution of the ovarian hormone will be undertaken.

Presence in Plants.

Loewe, Lange and Spohr (1927) found that willow catkins gave a positive reaction on castrate mice. Glimm and Wadehn (1928) indicated that it was present in yeast. Walker and Janney (1930) say that the oestrogenic substance in plants occurs in connection with the green pigment roots and that tubers were negative. A high concentration was found in plants which were undergoing rapid growth. Green parts of mature plants gave negative results. Sprouting oats caused the substance to appear in them. All ordinary plant products were negative. One should accept the results obtained with the plant material with caution. The number of animals used were usually quite small and a rather low standard for a positive smear was taken. The presence of some such substance in alder and willow catkins seems to have the best claim to recognition at the present time. Bauer (1931) has discussed the occurrence of oestrus-producing substances in plants and in invertebrates. He quotes various authors to show that it exists in a most extraordinary variety of places such as: sea-urchins and their eggs and sperms; in some worms, in the flowers of the water-lily, in sunflowers, and yeast; also in

some protozoa, in sea-anemones, hornets and spiders. Extracts of materials all produce oestrus in castrate rodents.

Animal Sources.

Loewe, Lange and Kaer (1929) found evidence of its presence in the female frog and in amphibia. Fellner (1925) using the growth of the uterus as criterion found it in the eggs of hens and fish. Doisy and co-workers (1927) were unable to confirm this, using the vaginal smear technique.

The Distribution in the Mammalian Ovary.

Much controversy has arisen as to the part of the ovary in which the hormone is present in greater amount and which part is the actual site of formation of the hormone. Doisy, Ralls, Allen and Johnston (1924) obtained much more potency from the liquor folliculi than from the residual tissue of the ovary, as also did Thayer and Doisy (1928). On the other hand, Dickens, Dodds and Wright (1925) and Parkes and Bellerby (1926) maintain that the concentration is equal between follicles and the rest of the ovary. The discrepancy may depend upon the type of ovary used and the stage in the oestrous cycle at which the source animal was killed. Parkes (1927) has shown that the oestrous cycle in the mouse will continue unchanged after complete destruction of all follicles, by means of X-Rays. This will occur whether the mouse is sterilized when immature or adult. He feels that this indicates that the Graafian follicle is not the essential source

of the oestrogenic hormone. However, Genther (1931) in guinea-pigs found that in half the cases, treatment with unfiltered X-rays caused marked fibrosis of the ovary, absence of sexual cycle and a continuous resting state. In the other half, whenever oestrus occurred, it was associated with at least one large follicle in the ovary. Zondek (1926) using the method of muscular implantation of fresh tissue, showed that the use of liver, spleen, uterus, pituitary, thymus, thyroid, suprarenal and testis all failed to produce oestrus in castrate female mice. He found that intermenstrually, the walls and fluid of the follicles contained the hormone, the concentration increasing to a maximum immediately before menstruation. He found still more in the corpus luteum in its early stages. Aschheim in the same year showed that uterine mucosa and human decidua were negative on castrate mice. They believe that the cells of the theca interna rather than the granulosa cells form the oestrin.

The presence of the oestrus hormone in the corpus luteum has been the subject of much controversy, all early workers believing that it was present there in large amounts. Some of them used the growth of the rabbit uterus as criterion so that the possibility of other than oestrus-producing substances (those that cause pseudo-pregnancy, for example) being involved in the effect must be considered. Okinschitz (1914) obtained negative results. Allen and Doisy (1923) also were unable to find any active substance in corpora lutea. They later found activity in hollow human corpora. Zondek (1926) found that early corpora

were very active but that as they matured at the time of menstruation, the value decreased rapidly. Parkes and Bellerby (1927) found that the fluid in the hollow corpora was active but that the solid lutein cells were not so. The presence of non-luteinized cells and follicular fluid in early corpora is probably the reason for the positive reactions obtained with this material.

The placenta has been known for a long time to possess oestrogenic activity on the castrate animal. Fels (1926) could not demonstrate it before the second month of pregnancy. Allen, Pratt and Doisy (1926) found it in placentae of the third month, and numerous workers have found it in placentae at a later stage. It was one of the chief sources of the hormone before the discovery by Zondek and Aschheim (1926) that the hormone was present in large amounts in pregnancy urine. The active substance has also been found in the foetal membranes, foetal blood, amniotic fluid and umbilical cord, but its concentration in the foetus itself is low. The species difference in the occurrence of the hormone in the placenta is of interest. It might be supposed that animals such as monotremes and marsupials which have no placental structure,and animals with very simple placentas, might have less hormone in these structures than those possessing a simpler structure. The anterior-pituitary-like hormone to be mentioned later which occurs in placenta, has been demonstrated only in humans and in the higher apes (Erhardt, 1929). Even Macacus appears to have none of it (Allen et al. 1931), but oestrin is present in both placenta and pregnancy urine of this species.

Presence in Body Fluids.

The presence of oestrin in the blood of non-pregnant females was shown by Loewe (1925) and by Frank and co-workers (1925, 1926). The latter followed the changes in its concentration occurring with the menstrual cycle in humans and used the concentration as a test of abnormalities of function of the ovaries. It is present in highest amount about the first day of menstruation after which it rapidly decreases. The menstrual blood contains it in higher concentration than the circulating blood. The hormone appears in larger amounts in the blood in pregnancy. The time of its appearance is in dispute. Frank found it in the fourth month, Zondek in the fifth and Fels (1926) after the sixth month. All are agreed that it increases rapidly in amount after this period. These effects were all studied on castrate animals. On the other hand, the substance which causes stimulation of the immature gonad appears very early. Aschheim (1926) found it in decidua before the placenta was actually formed at all.

Loewi (1925, 1926, 1927) showed the presence of small amounts of oestrus-producing hormone in non-pregnant female urine. It was highest in the inter-menstruum. The discovery by Aschheim and Zondek as mentioned above, of the enormous amount of active substance in human urine during the latter part of pregnancy, have made available a rich source of the hormone and great progress in its purification. Aschheim and Zondek and Veler and Doisy (1928)

showed that it disappeared very rapidly after parturition. The amount in the blood falls to zero in about 13 days; that in the urine at first increases, then decreases. It also appears in the milk. The placenta has been shown to act as a site of formation of the oestrogenic hormone during pregnancy. Waldstein (1929) and Probstner (1931) found that the blood of pregnant women after ovariectomy at about the 34th day showed a normal concentration of the hormone in the blood throughout the pregnancy. The amount excreted in the urine was, however, much less.

The presence of oestrin or oestrin-like substances in the male animal have been reported. Frank and Goldberger (1928) found evidence of activity in male blood. Laqueur and de Jongh (1928) showed its presence in small amounts in male urine. This was also found by Loewe and co-workers (1928). Fee, Marrian and Parkes (1929) were able to produce not only vaginal cornification but also uterine enlargement in castrate mice by the use of extracts from male urine. It was important to prove the uterine growth in addition to the vaginal reaction since it has been shown by Evans (1928) that deficiency of vitamin A in the diet leads to continuous vaginal cornification in castrate or normal animals. The occurrence of this cornification is thus not absolutely specific for the ovarian hormone. The source of the active substance in male is not known. Fellner (1921) claimed to have obtained positive results with testis but Allen and Doisy(1927) were unable to do so. The presence of oestrogenic substances in

plants might account for its presence derived from the food, but a definite decision cannot be made at present. The oestrogenic hormone has also been shown to be excreted in the faeces.

A few of the yields in terms of rat or mouse units from these sources are given below. The significance of the unit varies greatly with different methods of assay and so, this table is only an approximate statement.

TABLE IV.
(from Parkes, 1929)

Yields of Hormone from Various Sources.

Sources	Yield	
Whole ovaries:		
Cow (adult)	293	m.u./kg.
(immature)	73-350	m.u./kg.
Pig (adult)	219	m.u./kg.
(immature)	166-273	m.u./kg.
Liquor folliculi:		
Human	433-7000	r.u./litre
Cow	37-788	m.u./litre
Pig (avg.)	878	r.u./litre
Placenta:		
Human	192-2123	m.u./kg.
	400-700	r.u./kg.
Cow (maternal)	203-3200	m.u./kg.
(foetal)	143-782	m.u./kg.
Sheep	183-308	m.u./kg.
Female blood (human):		
Late pregnancy	1000	m.u./litre
Female urine (human):		
Pregnancy	4-10,000	m.u./litre
	470-1240	r.u./litre
Residual Ovarian Tissue:		
Pig	225	r.u./kg.
Cow	150-326	m.u./kg.
Pig	227-865	m.u./kg.
Corpora lutea (human):	3700	r.u./kg.
Pig (unsorted)	16	m.u./kg.
(solid)	none	
(tissue of hollow)	11	m.u./kg.
(fluid of hollow)	184	m.u./kg.
Male urine:	1-3	m.u./litre.
Plants:		
Willow catkins	200	m.u./kg.
Press yeast	30	m.u./kg.

The great variation in unitage from the same source indicates the uncertainty of quantitative recovery and of assay of the hormone at the time when this table was drawn up.

The Methods of Purification of Oestrogenic Substances.

The purification of the ovarian hormone waited upon the discovery of a suitable test object and of a rich source of supply. The vaginal smear of the female castrate rodent provided the former and the urine of pregnancy provided the latter.

A review of the methods of purification used prior to the discovery of the crystalline hormone by Doisy and by Butenandt in 1929 will be given first. The methods of preparation of crystalline preparations will then be described in detail. The chemical properties of the impure preparations have for the most part no bearing upon the true properties of the hormones so that they will be only mentioned where they appear to indicate the properties of the finally isolated products.

The Early Methods of Purification.

As mentioned above, Iscovesco (1912) working with ovaries, was the first to show that the hormone was extractable by fat

solvents. He was able to remove the proteins, most of the neutral fat and cholesterol. The active fraction accompanied the following steps:

(1) 95% alcohol.

(2) Dried and extracted with ether.

(3) Dried and again extracted with ether.

(4) Ether soluble dried and taken up in acetone placed in ice-box. Filtered.

(5) Acetone off. Dissolved in alcohol and cooled. Cold acetone and alcohol insoluble fractions, mostly cholesterol.

(6) The cold alcohol soluble material was dried and extracted with ether.

The activity (uterine-stimulant) was mostly in this last fraction. This use of a series of different fat solvents is, frequently still employed in the preparation of the hormone. Hermann (1913, 1915) was able to carry the purification somewhat further. He used part of the Iscovesco method. The original material was extracted with ether, the residue was extracted with acetone, then with ether and chloroform. These fractions were united and extracted with methyl alcohol. The cholesterol was thrown out by cooling, the methyl alcohol taken off and the material re-extracted with ether. His addition to the technique was the distillation of the final residue in high vacuum (0.06 mm. mercury) at an internal temperature of 193°C. (Compare Butenandt, 1929). His final product still gave the cholesterol reaction. It was an oil and contained only carbon,

hydrogen and oxygen, no nitrogen and sulphur. He believed that the pure substance was a cholesterol derivative. He used placenta as a source of material.

The question as to whether the substance was a lipoid or merely carried over by the lipoids into the lipoid solvents, was much discussed at this time. Frank (1917) carried out a series of investigations into the purification and properties of the hormone. He found that it resisted a temperature of 350°C., strong acids and alkalies hot or cold, without loss of potency. He found that it lost its activity on standing. He also concluded that the final product obtained by Hermann's method was not a pure substance. Frank was able to free the active fraction completely from cholesterol. He used uterine growth as a criterion of activity. Giesy (1920) introduced the distribution between 75 per cent alcohol and petroleum ether as a means of purification. The cholesterol is 26 times more soluble in the petroleum ether than in the alcoholic solution, while the hormone is relatively insoluble in the petroleum ether. He found that the activity was readily destroyed by oxidation, but not by heat alone. Most of the final products at this time consisted of yellow oils. In 1924 Doisy, Ralls, Allen and Johnston, using the vaginal smear as the criterion of activity and employing follicular fluid as the source, obtained more concentrated preparations. They removed cholesterol by the digitonin method. Doisy, Ralls and Jordan (1926) and later, Ralls, Jordan and Doisy (1926) published various modifications of their

extraction process. A summary of their final process is as follows:

> Liquor folliculi mixed with 95% alcohol proteins precipitated. Distill alcohol. Hydrolyze for short time in alcohol or water with gentle heat. Dissolve in water. Render alkaline with sodium hydroxide. Extract with ether three or four times. Wash ether with water. Distill off ether. Partition between 70% alcohol and petroleum ether. Distill alcohol solution to dryness in vacuo. Leach with redistilled ether.

They were able to reduce the solids per rat unit to 0.03 mg. Jordan and Doisy (1926) found that light inactivated the hormone. Even diffuse daylight caused considerable loss. The loss depended upon the solvent used. Hexane will cause a 50 per cent loss in two days. The presence of eosin and some other substances such as haematoporphyrin caused the inactivation by light to proceed at a more rapid rate; again a 50 per cent loss was sustained in a period of two days. In sunlight, in a quartz tube in alcohol with a little eosin added, one hour was sufficient for complete destruction; quinine was also very effective in hastening destruction. Ultra-violet light quickly destroyed activity (Allen and Ellis, 1925).

Glimm and Wadehn (1925) using placenta employed much the same technique as that of Hermann. They precipitated with lead acetate at one point, and got a product having a weight of 9.1 mg. per mouse unit. The precipitation of the hormone by this and other means is probably usually a case of adsorbtion on the precipitate, as shown by Gustavsen (1925).

Water Soluble Preparations.

In 1925, Zondek and Brahn, Laqueur and co-workers obtained an active water-soluble preparation of the active material. They used placenta and follicular fluid as sources of material. Later Zondek (1928), following the discovery by Loewe (1925) of oestrin in the urine of non-pregnant women, showed that it was present in large amount in the urine of pregnant women. He modified his technique slightly and used this as a source of hormone:

> He acidifies the urine with acetic acid and filters, concentrates in vacuo to half volume, and filters; then extracts with large amounts of ether. The ether residue is taken up in water and treated with hot alkali. The alkaline solution is extracted with ether and the ether residue is taken up in distilled water or dilute acetic acid.

Laqueur (1927) amplifies his method which Dickens, Dodds and Brinkworth (1927) had been unable to repeat:

> He boils the minced placenta with benzene; this only extracts about one-third of the hormone originally present in the placenta. He distills the benzene, extracts with 80% alcohol and takes off the alcohol. The residue is shaken with water acidified with HCl left in the cold for 12 hours and centrifuged or filtered through glass wool or asbestos.

He claims that in aqueous solution, the hormone is adsorbed by filter paper, talcum, and supra-norite. He called his substance

"menformon". It resisted digestive enzymes but was susceptible to oxidation. Lipschutz and co-workers have also prepared a water soluble material by a modification of Allen and Doisy's procedure. By repeated water to ether extractions, they obtained a material for which they claimed a weight of 0.00002 mg. for a mouse unit. These aqueous solutions lose all their potency in a few days. Biedl (1927) by electro-dialysis of an emulsion of ovarian lipoids, got the active substance to pass through the membrane to the negative pole. Dickens, Dodds and Brinkworth (1927) prepared a water soluble fraction by means of picric acid precipitation. They had some difficulty in getting uniform results and later, Allan, Dickens, Dodds and Howitt (1928) changed the method of extraction: They mince the placenta, add aqueous barium hydroxide and boil for two hours, concentrate and extract with butyl alcohol. The solvent is distilled and the residue extracted with ether. By further purification and removal of the barium, they succeeded in getting a water soluble preparation with a unit weight of 0.01 mg. They found the active material to be dialysable and to pass a porcelain filter.

Veler and Doisy (1928) and Thayer, Jordan and Doisy (1928) described improved methods for the preparation of the hormone with rather higher yields than before. They got a yield of 480-1240 R.U. per litre of urine. They were able to obtain an active aqueous solution and to reduce the weight per rat unit to below 0.001 mg. They point out that solubility in water is relative

since with such an active material, a very slight solubility as chemical solubilities go, results in a solution of high physiological potency. They obtained their clear aqueous solution by filtration through asbestos and found that some of the potency was retained on the filter. They emphasized the destruction of the hormone by peroxides formed in ether or petroleum ether on standing. In order to avoid these losses which become more serious as the preparation is further purified, they used peroxide free ether and distilled off the ether as soon as possible. They found the hormone stable indefinitely in alcohol for as long as four and a half years. Aqueous solutions seem to keep quite well as do crude ether extracts. A petroleum ether solution lost four-fifths of its potency in 3 years. The purified aqueous and alcoholic solutions kept quite well in the absence of oxidising agents. They warned against the concentration of the peroxides by the distillation of large volumes of solvent and the consequent great, destructive effect on the hormone in the residue. The ether solution when crude, may keep well but the peroxides which have formed on standing will destroy the hormone as soon as purification is attempted. They tested for peroxides in their solvents (ether and petroleum ether) with potassium iodide. They also tried the effect of adding a strong, reducing agent such as hot, alkaline glucose to the solvent to destroy the peroxides. This seems to work satisfactorily, but anti-oxidants like hydroquinone do not. Marrian (1929) described a method which he later modified and

which will be described in more detail later.

The Evidence for the Existence of a Number of Ovarian Hormones.

Up to this point, it has been assumed that only one or at the most, two hormones exist connected with the female sex organs. The evidence that there are more will be considered at this point. The isolation of a number of crystalline hormones capable of producing oestrus changes in the castrate with varying degrees of effectiveness will be discussed later.

The work of Zondek and Aschheim (1926, 1927), Smith and Engle (1927), and others has clearly established the existence of an ovary-stimulating hormone in the anterior pituitary. This is inactive on the castrate animal but is effective in causing the development of the immature ovary and accessory sex organs. This hormone is generally believed to regulate the cyclic changes in the ovary, causing it to produce the oestrus hormone. It also is connected with the production of ovulation. Hormones of this type have been shown by Aschheim and Zondek (1926) to be present in large amounts in the urine of pregnant women. They have also shown that implantations of decidua or placenta are effective in

producing premature maturity in mice.

Wiesner (1930) and Collip (1930)abc have succeeded in preparing extracts from the placenta which are active in this respect.

This type of substance is precipitated by alcohol destroyed by digestive enzymes and completely inactive on the castrate. It is not related chemically to the oestrogenic hormones which are the subject of this thesis. Philipp (1930) and Collip (1930) believe that the substances of this type occurring in human placenta are formed there and are not stored anterior pituitary hormone.

As mentioned above, the early workers considered the corpus luteum as the only endocrine gland of the ovary. The work of Corner (1929) and Hisaw (1929) has definitely established the presence of hormones other than those of the oestrus-producing type in the corpus luteum. Recently Fevold, Hisaw and Leonard (1932) have divided the corpus luteum extract into three fractions having different physiological effects.

The presence of a substance in human placenta soluble in alcohol, which acted in very small doses by mouth in producing oestrus in the immature rat, but which was almost inactive on the adult castrate rat when it was injected, was demonstrated by Dr. Collip in 1930. The substance could not be extracted from acid aqueous solution by repeated extractions with ether. It thus differed in chemical properties and physiological properties from

the then-known properties of oestrin. Zondek (1930) showed that it was impossible to extract the larger part of the oestrus-producing hormone from the acidified urine of pregnant mares with chloroform. In the case of most other species, the hormone was almost all extracted from acid solution by repeated use of chloroform or ether. This also indicated a difference in chemical properties, whether the hormone was merely bound to some other substance which prevented its extraction by fat solvents, or whether the substance remaining behind was a different chemical form of the hormone. It is with the elucidation of the chemical and physiological differences between different forms of the oestrus-producing hormone that this thesis is concerned.

Nomenclature.

The early workers referred to the active substance as 'the ovarian hormone' or 'the female sex hormone' (Frank, 1929). Glimm and Wadehn (1925) called it 'feminin'. Allen and Doisy (1927) used the term 'genital growth hormone'. 'Follicular hormone' and 'folliculin' have been used by Courrier (1924) and others. Laqueur (1921, 1925) called his water-soluble preparation 'menformon'. Loewe (1925) used 'thelytropine' to include both

the oestrus-producing hormone and that of the corpus luteum; the former, he calls 'thelykinin'. Blotevogel, Dohrn and Poll (1926) employ the term 'tokokinin'. Parkes (1929) prefers the use of 'oestrin'. Butenandt (1920) called his impure preparation 'progynon' and also used this term for the crystalline substance isolated by him. He now uses the term 'follicular' or 'oestrus hormone' and for the second crystalline product first isolated by Marrian (1930), the term 'hormone-hydrate' since it differs from the first compound by one molecule of water. Marrian (1931) first used the terms 'di-oxy-' and 'tri-hydroxyoestrin' for the two crystalline compounds. He now employs the term 'keto-hydroxy-oestrin' instead of 'di-oxy-'. Doisy (1930) gives the name 'theelin' to the compound first isolated by him and 'theelol' to the tri-hydroxy compound isolated by him in 1931.

The Preparation of Crystalline Oestrogenic Hormones.

At the International Physiological Congress in Boston in August, 1929, E. A. Doisy demonstrated a crystalline preparation of the female sex hormone. Shortly after, Butenandt (1929) independently described a crystalline hormone. A method for its preparation was described by both these authors in detail in 1930. In the same year, Laqueur and co-workers also reported the preparation of a crystalline substance by a different method. Marrian (1930) described a method of preparation for a crystalline substance which he felt to differ from that of Butenandt. He questioned whether any of the crystalline substances were the pure hormone. D'Amour and Gustavson (1930) using a slightly different method, were able to prepare a compound having the same physiological potency as that of Doisy.

The various methods for the preparation of the crystalline substances will be given in detail. The section after that on preparation will deal with the physical and chemical properties of these substances.

The Preparation of Theelin from Pregnancy Urine (Doisy)

Doisy's method for the extraction of the crystalline hormone was first given in detail in 1930 (Doisy, Veler and Thayer, 1930) and later modified in the same year (Veler, Thayer and Doisy, 1930).

They first used olive oil as an extraction medium for the urine. They state that the urine must be acid when extracted since about 75 per cent of the hormone may be left in the urine if extracted on the alkaline side. They extracted the oil with ethyl alcohol and from there divided the preparation into several fractions. Much of this fractionation, they discarded in their second paper. Here they employed chloroform or butyl alcohol as the original extraction medium. A continuous liquid extractor for use with large volumes of urine was described for each solvent. Butyl alcohol gave the largest yields of hormone. The capacity of their extractor was 25-30 gallons in 24 hours.

The butyl alcohol was distilled, the residue extracted with benzene, then cooled and the benzene poured off from the tar (loss in tar 3.2%); this leaching with benzene was repeated several times. They then distilled off the benzene and extracted the residue with hot 0.5N sodium hydroxide (residue discarded, loss 4.2%). In earlier preparations, they felt that losses were encountered with hot alkali so they transferred the hormone to alkali by addition of a small volume of butyl alcohol and a large volume of petroleum ether. This was then extracted with cold 0.5N alkali. In the later preparations they set boiling 0.5N NaOH without loss. The alkaline solution was extracted repeatedly with ether. The pH during this extraction was between 9 and 10. This does not extract all the hormone but they felt that the purification obtained compensated for this loss. They again cautioned against the presence

of peroxides as destructive agents. The ether was distilled, the residue steam distilled and then extracted with hot 0.25N NaOH (loss in residue 1%). The solution was filtered and extracted with ethyl ether. The ether solutions were washed with sodium bicarbonate, hydrochloric acid and water. The ether was distilled and the residue extracted with cold 0.25N NaOH (loss in residue 9%). This was extracted once with ether. The next five extractions were combined and the ether distilled. The residue was dissolved in alcohol and hot water added. The hormone usually precipitated in a semi-crystalline form. It was dissolved in hot butyl alcohol and precipitated with petroleum ether. It was recrystallized from 20 per cent aqueous alcohol.

The essential steps in this process depend upon the extraction of the hormone from alkaline solution with ether. It is in this paper that they gave the name theelin to the crystalline hormone. They presented evidence that the crystals were actually the hormone.-

(1) The potency exceeded any non-crystalline preparation.

(2) Several preparations of the crystals had the same potency.

(3) Careful microscopic examination revealed only one type of crystal.

(4) Repeated recrystallization did not diminish the potency which was about 0.38 γ per rat unit.

The Preparation of Crystalline Follicular Hormone. (Butenandt,1930).

Butenandt first used placenta as original material but was unable to proceed further than a very active oil; his best potency was about 2.0γ. He then used pregnancy urine extracted after acidification with various fat solvents. The solvent is distilled and the oil so obtained is used for further purification. The summary of his method is presented, together with the weight of each fraction which corresponds to a mouse unit.
The gamma is one thousandth of a milligram.

TABLE V.

Summary of Butenandt's Method.

Raw Oil
35γ

Distribute between 50% methyl alcohol and petroleum ether

Alcohol Phase — Petroleum Ether
(Benzoic Acid, Cholesterol)

Dilute 3-4 times with water.
Extract with ether.
Ether distilled.
Residue: 7-10γ
Little loss.

Distribute between 70% ethyl alcohol and benzene

Benzene Phase — Alcohol Phase
Distill solvent. Nitrogen containing acid.
Residue: 2-3γ (Decomposition 305°C.)
15-40% activity remains in alcohol phase.

Treat with dilute HCl. Extract with ether.
Wash ether with Na_2CO_3.

Carbonate Solution — Extract ether with 1N NaOH

Alkaline Solution — Alkali Insoluble
Acidify to congo red. (Pregnandiol M.P. 235° C.)
Extract with ether.
Residue: 0.5-0.7γ

Fractional distillation in high vacuum.
(0.02-0.03 mm.Hg. at 130°-200°C.)

Impure crystals: 0.12-0.14γ — Recrystallized from
Recovery of original ethyl acetate.
potency 64%. 0.1-0.12γ

This method also makes use of the acidic properties of the hormone in forming ether soluble salt in alkali but adds the use of high vacuum distillation. A mercury vapour pump is used to obtain the vacuum of 0.02-0.03 mm. mercury. The material is heated in an air bath. For the first 12 hours the temperature is kept at 110°-115°C.; an oil distills over which is washed out of the retort with ether at the end of the period. For the next 60 hours the temperature is slowly raised to 220°C. From 130° C. on crystals deposit on the sides of the retort. They are washed with cold ether and dissolved out of the retort with acetone. The acetone is distilled, the crystals dissolved in ethyl acetate and the last impurities precipitated with an equal volume of warm petroleum ether. Recrystallization is performed from ethyl acetate. The mouse unit is not the same as Doisy's rat unit. The differences will be discussed under the section on assay.

Other Methods of Preparation.

D'Amour and Gustavson (1930) precipitate the anterior pituitary hormone from the concentrated urine with alcohol, evaporate off the solvent, extract with benzene. They then perform a series of extractions with diminishing volumes of 5 per cent sodium hydroxide, acidifying till just alkaline to phenolphthalein and extracting with ether. They then distill at a temperature of 130°-140°C. and a pressure of 0.02 mm. They agree with Doisy as to the weight per rat unit.

Laqueur and co-workers (1930) describe a method for the preparation of crystalline menformon. The principle this depends on the adsorption on Fuller's earth. After freeing by heating with benzene, an alkaline solution is obtained and extracted with benzene; this is repeated several times and crystals obtained.

In 1929 Wieland, Straub and Dörfmuller reported the isolation of a crystalline material which however had much less potency than the other crystalline preparations and was probably not a pure substance. However later work (Butenandt, 1930) showing the presence of a low potency crystalline substance may explain their low potencies.

The Preparation of a Second Crystalline Hormone from Pregnancy Urine. (Marrian, 1930)

In 1929, Marrian elaborated a process for the concentration of the female sex hormone from pregnancy urine. In 1930, he purified this concentrate still further and obtained a crystalline product. A modification of his method was the one which was followed in most of the preparations of crystalline substances from placenta in the experimental part of this thesis.

Marrian extracted acidified pregnancy urine four times with ether, washed the ether with water, evaporated to dryness and stored the preparation in alcohol at 0°C. When sufficient concentrate had accumulated, he evaporated off the alcohol. He obtained a yield in the crude extract of 18,800 mouse units per litre of urine. The dried extract was dissolved in 5 per cent

aqueous potassium hydroxide and heated on a boiling water bath for about 2 hours. Then carbon dioxide was passed into the mixture for 12 hours. The carbonate solution was extracted with ether twelve times. The ether extracts were washed with hydrochloric acid and water and evaporated to dryness. He lost about 40% of the potency in these steps. The residue was dissolved in acetone and chilled (0° C. for 12 hours). This removed an inactive crystalline material which Marrian discovered in 1929. It melted at 233°C. and is a di-hydroxy alcohol. It is identical with the 'pregnandiol' of Butenandt. The acetone solution was immersed in an ice-salt freezing mixture for one hour and then filtered. The residue was washed with cold acetone and then dissolved in hot chloroform-alcohol solution and the treatment with cold acetone repeated. The acetone was evaporated and the residue dissolved in 50% alcohol. This was boiled for 5-10 minutes and chilled over night. The solution was filtered and the residue re-boiled with 50 per cent alcohol. This was repeated four times in all and the combined filtrates evaporated to dryness. The residue was dissolved in a small volume of alcohol and a large volume of ether added. The ether was extracted ten times with aqueous 5 per cent KOH. The alkaline solution was rendered strongly acid with HCl and extracted eight times with ether. The ether extracts were washed and evaporated to dryness. He failed completely to extract any potency from alkaline solution with ether when he tried to do this instead of passing carbon dioxide in first in the

step mentioned above. This is in contrast to the results of Doisy with theelin. The next step in the purification was the solution of the residue in a small volume of alcohol (0.75 cc.). About 10 cc. of ether were added and a granular precipitate was thrown down. The mixture was chilled to -15°C. and filtered rapidly with suction. The precipitate was washed with cold alcohol-ether mixture to remove the more soluble oily material. This precipitate was light brown and semi-crystalline. It was dissolved in alcohol and boiled for 30 minutes with 'norite'. This was repeated. The material was recrystallized from 57 per cent methyl alcohol or from ethyl acetate. The total loss of potency in the process was about 40 per cent. He compared his product with those of others, questioned its identity with theirs and doubted whether any of the crystalline compounds are the pure hormone. The potency of his first crystals was 0.12γ per mouseunit but this is not comparable with Butenandt's unit.

Other Preparations of the Second Crystalline Substance.

Preparation of a similar substance by Butenandt (1931) established the existence of two distinct crystalline hormones. He also worked out the relation between the two. This will be discussed under the chemical properties. His method is similar to that for the preparation of his first substance (See above p.53), down to the distribution between alcohol and benzene; that is to say,

he distributes the crude oil from pregnancy urine between 50 per cent methyl alcohol and petroleum ether, dilutes the alcohol with water and extracts with ether, then evaporates the ether and distributes between 60 per cent ethyl alcohol and benzene. This second compound, unlike his first, remains in the 60 per cent alcohol. The alcohol phase is treated with dilute HCl and extracted with ether, the ether shaken with sodium carbonate and extracted with sodium hydroxide. This is acidified and extracted with ether. The ether is distilled, the residue dissolved in alcohol, precipitated with ether as in Marrian's process and recrystallized. Marrian (1932) has very recently published a modification of his method which makes it more nearly like that of Butenandt. For the last step he precipitates the hormone from aqueous alkali by carbon dioxide as in the first step and recrystallizes for dilute alcohol.

Doisy and Thayer (1931) were led by their inability to extract all the activity from alkaline solutions with ether (compare Marrian who was unable to extract any of it in this way), to investigate the alkaline solution which contained this residual potency. They chose the step where the hot sodium hydroxide extract is extracted with ether the second time. The alkaline solution is faintly acidified with hydrochloric acid and heated to drive off the ether and coagulate the precipitate. The precipitate is filtered and dissolved in 95 per cent alcohol. This is evaporated slowly and a semi-crystalline mass results on cooling. The

residue is recrystallized once or twice in this manner and then boiled with 'norite' in alcoholic solution, filtered and concentrated. The hormone is precipitated by the addition of three volumes of water to the alcohol and then recrystallized from alcohol. He believes that the recrystallization from alcohol suffices to remove all the 'theelin'. He calls this new compound 'theelol'. He expresses the belief that Marrian's preparation was a mixture of the two.

Skarrzynski (1931) prepared a crystalline hormone according to Marrian's method but claimed that it had the melting point and absorption curve of Butenandt's keto-hydroxy form. He believes that the hormone is only adsorbed on the crystalline compound.

The above methods of preparation will be discussed after the chemical properties of the products have been described. One cannot compare the various potencies obtained by different authors because of the great differences in assay technique.

The Physical and Chemical Properties of Crystalline Oestrogenic Substances.

Physical Properties.

At least two forms of the female sex hormone exist. One form will be given the name 'keto-hydroxy' (Marrian and Haslewood, 1932). This includes Doisy's 'theelin' and Butenandt's 'hormone' and the other, 'tri-hydroxy'. This includes Doisy's 'theelol' and Butenandt's 'hormone hydrate'.

(1) Crystalline Form.- Doisy (1930) described two crystalline forms of his 'theelin'; one a leaflet which appeared on distilling ether to dryness, and the other a rhomboid platelet which appeared when the leaflet crystals were crystallized from aqueous alcohol. He also noted that the substance tended to form thick plates when crystallized slowly from alcohol. Butenandt (1931) described many crystalline forms according to the solvent and rate of crystallization. From ethyl acetate he obtained rhombic tabular plates. From dilute alcohol, the form differed according to rate of crystallization. From very dilute alcohol, it appeared as small, spindle-like, very refractile leaflets; by very slow crystallization from the same medium, as very long (1-2 cm.) branching crystals with a mother-of-pearl-like sheen. The crystals are colorless. Slawson (1930) has given a crystallographic description of 'theelin'. He says, "The crystals are

monoclinic with a distinct development to the side pinacoid and have a rhomboid outline with included angles of 112° and 68°. The angle of the rhomboid in many cases is truncated by a small crystal face. This variation in outline is superficial. The crystals are optically negative, the indices of refraction are α-1.520, β -1.642, and γ -1,692, and 2V is 50°-55°....The obtuse bisectrix is perpendicular to the side pinacoid.....The extinction angle with the longer edge of the crystals is 12° in the direction of the obtuse angle." The crystals show strong birefringence. 'Theelol' (Slawson, 1931) bears a very close relation to 'theelin' in all its crystalline properties: it is monoclinic, forming rectangular parallelopipeds, the crystals are optically negative, the α index of refraction is 1.533, the others are similar to 'theelin'. The extinction angle measured on one face is 5.5° less than 'theelin' by 7° . If the crystals lie on the other face the extinction is parallel.

(2) Melting Point.- These are very difficult to compare since most are uncorrected and the various authors use different methods. If not marked in the text, they are uncorrected melting points.

(a) The Keto-hydroxy Form: Veler, Thayer and Doisy (1930) give the melting point as 243.5°C. (uncorrected) and say that it melts with decomposition. Later, Thayer, Levin and Doisy (1931) state that complete melting occurs at 247°-249°C. Using calibrated short stem thermometers, they get 256.2°C. (uncorrected). With

preliminary partial melting some degrees lower, their first melting point was the point at which a few oily droplets appear. Butenandt (1930) and Laqueur (1930) both report melting points of 240°C. (uncorrected) and Butenandt (1930) gives the corrected value at 250°-251°C. At first, Butenandt used the term 'decomposition point' but later, preparations melted apparently with only slight decomposition. Some charring always takes place a few degrees below the melting point.

(b) The Tri-hydroxy Form: Marrian (1930) states in his first paper that from 222°C. on,the substance showed darkening and shrinking and decomposed at 256°-260°C. melting as it did so. In his second paper, Marrian (1930) gives the melting point as 264°-266°C. (uncorrected) with preliminary darkening at 255°C. Butenandt (1931) obtained a melting point of 268°-269°C. (uncorrected) with both his own preparation by Marrian's method and Marrian's own preparation. A mixed melting point gave the same value as the two separately. Thayer, Levin and Doisy (1931) give a melting point for their 'theelol' of 273°C. (with a long stem thermometer uncorrected). With a short stem thermometer it was 281°C. Butenandt later (1931) by a method of his own, was able to prepare a substance apparently the same as Marrian's in composition, but with a melting point of 276°C. (uncorrected). It still showed slight decomposition with melting. A mixed melting point with the earlier preparation melting at 268°C. showed no lowering of melting point. In Marrian and Haslewood (1932)

Marrian's latest preparation gives a melting point of 279.5°-280.5°C. (uncorrected) using a short stem thermometer.

(3) Solubility.-

(a) The Keto-hydroxy Form.- The crystalline hormone (Butenandt, 1931) is easily soluble in alcohol, acetone, benzene; soluble with more difficulty in ether and ethyl acetate, and soluble with difficulty in petroleum ether. It can be precipitated from alcohol or acetone by the addition of water, and from ethyl acetate, chloroform and benzene, also from butyl alcohol (in which it is very soluble) by the addition of petroleum ether.

The water solubility of the hormone was the subject of much controversy before the isolation of the crystals. The solubility of the hormone in water in neutral solution is very slight. On the basis of highest physiological potency obtainable, Butenandt (1931) gives a solubility of 1.5 mg. per 100 cc. The hormone is very soluble in cold aqueous alkali. It cannot be readily extracted from alkali by ether. It is precipitated from alkaline solution by carbon dioxide (Marrian, 1930) or by mineral acids. This behaviour has been taken to mean that the hormone is weakly acidic in nature, forms water soluble salts with the alkalis and is displaced from these salts by stronger acids. The group responsible for the acidic character is discussed later.

(b) The Tri-hydroxy Form: Marrian (1930) states that his substance showed a fairly low solubility in all fat solvents.

In ether, the solubility was very low; in methyl and ethyl alcohols, chloroform and acetone, somewhat greater. It was easily soluble in pyridine. In 5 per cent aqueous potassium hydroxide, it dissolved slowly but completely in the cold and was precipitated from the solution by the passage of carbon dioxide through it. It was insoluble in sodium carbonate. Doisy (1931) says that the solubilities of 'theelol' are similar to those of 'theelin'. The former, however, is not extracted to any extent from aqueous alkali by ether. The hydrated form is said to be more soluble than the hormone in water, but no figures are given. (Butenandt and Hildebrandt, 1931).

(4) Optical Activity.-

(a) The Keto-hydroxy Form.- Doisy gives a specific rotation in 95 per cent alcohol as $\alpha_D = +156^\circ$.

(b) The Tri-hydroxy Form.- Marrian (1930) gives for his compound (using the mercury green line) the rotation $\alpha 5461 = +38.5^\circ$ in pyridine. His latest preparation gives $\alpha 5461 = +41.9^\circ$. Butenandt obtained $\alpha_D = +34.4^\circ$ and $\alpha 5461 = +38.5^\circ$ in the same solvent. There was no difference in rotation between Marrian's preparation melting at 268°C. and his own melting at 276°C. However, Doisy for theelol in alcohol gives the value $\alpha_D = +61^\circ$. After standing for 22 hours, the rotation was unchanged. Very recently, Marrian and Haslewood (1932) have determined the rotation of the compound (M.P. 280°C.) in alcohol and obtain the value $\alpha 5461 = +76^\circ$.

(5) Ultra-Violet Absorption.- Butenandt (1931)[a] showed that the hormone in alcohol, using the mercury vapour lamp and the photo-electric method, gave an absorption maximum in the ultra-violet at 283-285 mm. The absorption spectrum for the other compound has not been done.

(6) Other Physical Properties.- Bernal (1932) determined the crystalline form of both 'keto-hydroxy oestrin' and 'tri-hydroxy oestrin'. He found that they were very similar. He also performed an X-ray analysis of the crystals and determined the cell-dimensions. These also showed great similarity between the two forms and a resemblance to anthracene or more probably phenanthrene, rather than pregnandiol or sterols. The lattice constants suggest molecules about 11 x 7.5 x 4.2 Å. Adam, Danielli, Haslewood and Marrian (1932) have found that tri-hydroxy oestrin triacetate and tri-hydroxy oestrin monomethyl ether form stable monomolecular films on water, which in condensed form, indicate an area for the molecule of 32.5 Å (compare the dimensions given by Bernal 4.2 x 7.5 = 31.5 Å). This, they believe to indicate that oestrin is not a sterol derivative but is possibly a partially reduced phenanthrene or anthracene derivative. For a discussion of the chemical derivatives involved in the above and the theory that oestrin is related to the sterols, see the section on chemical properties below.

Chemical Properties.

(1) Colour Reactions.- Doisy reports that both 'theelin' and 'theelol' give a positive Millon's test. Butenandt reports a negative Salkowski and Liebermann-Burchard reaction. Concentrated sulphuric acid gives a yellow-red colour. Marrian has also observed this colour with his substance and says that it is indistinguishable from that given by crude bile acids. Lipschutz's oxycholesterol reaction gave a delicate rose-pink colour. Rosenheim's trichloracetic acid reaction was negative. Kober (1931) has modified the colour with sulphuric acid and used it as a means of colorimetric determination of the hormone.

(2) Chemical Structure.-

i. Qualitative analysis.

(a) The Keto-hydroxy form.- Dingemanse, de Jongh, Kober and Laqueur (1930) found no halogens or phosphorus. Doisy (1931) found no nitrogen. Butenandt (1931)[a] found no nitrogen, sulphur or phosphorus. The hormone contained only carbon, oxygen and hydrogen.

(b) The Tri-hydroxy form.- Marrian could detect no nitrogen in his substance. Doisy for 'theelol' failed to detect anything but carbon hydrogen and oxygen.

ii. Quantitative Analysis.- A table of the carbon-hydrogen analyses of the various substances is given below together with some other chemical properties.

TABLE VI.

	Pregnandiol		Keto-hydroxy		Tri-hydroxy			
	Marrian	Butenandt	Doisy	Butenandt	Marrian	Doisy	Butenandt	Marrian & Haslewood. (1931)
C.%	78.08	78.64	79.69	79.68	74.76	75.12	74.75	74.95
H.%	11.18	11.33	8.49	8.21	8.39	8.38	8.33	8.27
OH	2	2	1	1	3	3	3	
C=O	0	0	1	1	0	0	0	
Mol.Wt.	284	#320	274	275	277	295	-	
Iodine No.	0	0	95.2	-	-	86.7		
Formula	$C_{20}H_{34}O_2$	$C_{21}H_{36}O_2$	$C_{18}H_{22}O_2$ or $C_{17}H_{20}O_2$	$C_{18}H_{22}O_2$	$C_{18}H_{24}O_3$	$C_{18}H_{24}O_3$	$C_{18}H_{24}O_3$ or $C_{17}H_{24}O_3$	

Calculated from the formula

The carbon-hydrogen determinations were made by the usual micro-method. The direct molecular weight determinations were done by Rast's camphor method.

The exact empiric formulae were in doubt for a long time and Butenandt is still doubtful whether there are 17 or 18 carbon atoms. The formula $C_{18}H_{22}O_2$ has gained most general acceptance for the keto-hydroxy form.

There are thus two oxygen atoms in the molecule. The compound isolated by Marrian (tri-hydroxy oestrin) on the other, on analysis and molecular weight determination, gives an empirical formula of $C_{18}H_{24}O_3$.

iii. Group Analysis: Hydroxyl Group.

(a) The Keto-hydroxy form.- Butenandt was able to prepare a crystalline mono-acetate (M.P. 126° C. uncorrected) showing the empirical formula $C_{20}H_{24}O_3$. This shows that one of the oxygen molecules is a hydroxyl group. Doisy prepared an acetate and believed that it was a di-acetate and, therefore, that the formula of 'theelin' was $C_{18}H_{21}(OH)_2$. Later, however, on analysis of the acetate, he was to prove that it was a mono-acetate and so, that only one hydroxyl group occurred. Butenandt also got occasionally a di-acetate and believed that this indicated enolization of the carbonyl group (see below). Butenandt was able to prepare a mono-benzoyl derivative but not a mono-methyl ether. Doisy prepared a mono-methyl ether from 'theelin' (M.P. 165° C.). Marrian and Haslewood (1932) also obtained a mono-methyl ether from the keto-

hydroxyl form (M.P. 164-167° C.). Both the mono-methyl ether and the mono-ester were insoluble in aqueous alkali. Saponification of the acetate gave the original compound unchanged.

(b) The Tri-hydroxy form.- Marrian's product on the other hand, gave a tri-acetyl derivative indicating the presence of three hydroxyl groups. He hydrolyzed the ester and identified the product as his original tri-hydroxy compound. Doisy was also able to prepare a tri-acetate from 'theelol' and to hydrolyze it and obtain again the original'theelol'. Doisy was able to distill the tri-acetate without decomposition in high vacuum; in the distillate he recovered the tri-acetate unchanged. On treatment with di-methyl sulphate, Doisy prepared a mono-methyl ether from 'theelol' (M.P. 154.8°C.uncorrected). Butenandt also obtained a mono-methyl ether by the use of diazomethane on his 'hormone hydrate'. This indicates that only one of the hydroxyls in the tri-hydroxy compound is acidic in nature. That this is so, is also indicated by the electrometric titration of the compound by Marrian. He found that the substance had an equivalent weight of 268-298 which is close to the molecular weight and that it showed only one pK value which was at about 10.8. From this he believes that the acidic hydroxyl is phenolic in nature. This is also suggested by the presence of a positive Millon's test, the formation of a blue colour with diazotised p-nitraniline and a yellow colour with concentrated nitric acid. Doisy also obtains a positive Millon's test with both 'theelin' and 'theelol' and concludes that the acid nature of both is due to a phenolic hydroxyl.

The Ketone Group.

(a) The Keto-hydroxy form.- Only one of the oxygen groups was accounted for as a hydroxyl group and Butenandt demonstrated the presence of a ketone group in his hormone by the preparation of an oxime (M.P. 230°C.(uncorrected) with the use of hydroxylamine in acetic acid solution. He was later able to prepare a semi-carbazone also. Doisy prepared an oxime from 'theelin'.

Butenandt because of his failure to prepare a mono-methyl ether regarded the acidic nature of the hormone to be of the nature of a keto-enol tautomerism, the addition of alkali causing the enolization of the C=O to an acidic hydroxyl. In his early work, Butenandt attributed this acidic nature to a lactone ring formation. He later abandoned this view for that of the keto-enol tautomerism.

(b) The Tri-hydroxy form.- All the oxygen atoms are accounted for as hydroxyls in this form. Butenandt was unable to prepare an oxime. This he regards as indicating that the keto-enol tautomerism existing in the hormone has been lost by hydration, the enol form having been stabilized.

The hydrated form still shows acidic properties like the hormone itself, however. This seems to be against the acidic behaviour of even the keto-hydroxy form, being due to a keto-enol tautomerism.

In connection with this question, Doisy believes that the

insolubility of the mono-methyl ether and the mono-acetate of 'theelin' and 'theelol', together with the fact that both 'theelin' and 'theelol' give a positive Millon's test, is strong evidence that the acidic group in both forms is a phenolic hydroxyl and that it is a salt formation with this group which accounts for the alkali solubility of both forms. On this basis, the phenolic hydroxyl would not be involved in the change from one form to the other. Marrian and Haslewood (1932) have recently brought forward evidence that the acidic nature of the compounds is due to the phenolic hydroxyl. They point out that it might be the enolic form of the keto-group which formed the mono-methyl ether and gave the Millon's test, and that consequently Doisy's argument is not final. Now Butenandt (1931) showed that it was possible to convert the tri-hydroxy form into the keto-hydroxy form by heating in a high vacuum with a dehydrating agent (Potassium acid sulphate). This occurred with the loss of a molecule of water and clearly established the relationship between the two forms. Marrian and Haslewood (1932) formed the mono-methyl ether of the tri-hydroxy form (M.P. 162.5-164°C. $\alpha 5461 = +29.9^{\circ}$). They also formed the mono-methyl ether of the keto-hydroxy form (M.P. 164-167°C $\alpha 5461 = +171^{\circ}$). He then heated the ether of the tri-hydroxy form in a high vacuum with potassium acid sulphate and obtained a dehydration product (M.P. 163-167°C. $\alpha 5461 = +173^{\circ}$). This compound had a similar crystalline form to and gave no change on mixed melting point with the mono-methyl ether of the keto-hydroxy form.

This proves conclusively according to him, that the conversion of tri-hydroxy to 'keto-hydroxy oestrin' is accompanied by the elimination of water between the two non-acidic hydroxyls and that the acidity of the keto-hydroxy form is due to a phenolic hydroxyl and not to a keto-enol tautomerism. Butenandt (1932) has also shown that contrary to his previous view the acidity and hence the alkali-solubility is due to the phenolic hydroxyl, since the compounds in which this group is blocked up (esters) are not alkali-soluble whereas those in which the carbonyl group alone is blocked up are still alkali-soluble.

iv. Structure of the Remainder of the Molecule.

Butenandt (1931)[a] by the use of catalytic hydrogenation of the acetate of the keto-hydroxy form obtained a hydride of composition $C_{18}H_{30}O$. The presence of the single oxygen atom after hydrogenation is not explained. He regards it as the oxygen of the original hydroxyl group obtained by hydrolysis of the acetate in the course of hydrogenation. The $C{=}O$ group has been replaced by CH_2 and three double bonds have been filled in by hydrogen. The hydride is inactive physiologically and is insoluble in water. He goes on to say that if the phenolic nature of the hormone is regarded as established, then the hydride may be considered as being formed by replacement of the phenolic hydroxyl by hydrogen and the saturation of four double bonds by hydrogen. The double bonds might be present in one benzene ring and one aliphatic double

bond or in four hydro-aromatic rings with three double bonds. Doisy found that there was only one double bond in 'theelin' which was capable of taking up iodine. The nature of the ring or rings in the compound is not known. If they are hydro-aromatic the hormone might be related to the sterols and the bile acids, and more closely related to 'pregnandiol'. This last compound was first isolated from the urine of pregnancy by Marrian in 1929 Butenandt gave it the name 'pregnandiol'. It melts at 233°C. and is inactive physiologically. It is relatively insoluble in alcohol. It is fairly soluble in boiling acetone. It is not precipitated by digitonin. The Liebermann-Burchard reaction gives a deep brown-red colour. Butenandt proved that it was a sterol derivative of the allo-cholesterol series (Butenandt, 1931)[c] and might have the formula:

As noted above under the section on X-ray crystallography, Marrian and co-workers (1932) now regard the hormone as being related to phenanthrene or anthracene rather than to the sterols and 'pregnandiol'. Further work is needed to clarify this situation.

A table of the derivatives of the two forms of oestrin is given below:

TABLE VII.

Table of properties of derivatives of female sex hormones.

	M.P. (uncorrected) °C.	Specific Rotation	Alkali Solubility
Theelin (Doisy):	248 . .	+156°(Dline) alcohol	Yes
Mono-acetate	125.3	-	No
Mono-methyl ether	163-165	-	No
Oxime	229	-	No
Hormone (Butenandt):	250	+156°(Dline) chloroform	Yes
Mono-acetate	126	-	No
Bromacetyl	133-147	-	No
Benzoyl	211	-	No
Oxime	230	-	Yes
Semicarbazon	245	-	Yes
Hydride	104	-	No
Keto-hydroxy (Marrian):	#242-246	+188°(5461) alcohol	Yes
Mono-methyl ether	#164-167	+171°(5461) chloroform	No
Oxime of mono-methyl ether.	#182-187		No
Tri-hydroxy (Marrian):	#279.5-280.5	+ 41.9°(5461) pyridine + 76° (5461) alcohol	Yes
Tri-acetate	120-122	-	No
Mono-methyl ether	162.5-164	+29.9° (5461) pyridine	No
Hormone Hydrate (Butenandt)	276	+34.4° (Dline) pyridine +38.5° (5461) alcohol	Yes
Mono-methyl ether	168	+34.7° (Dline) pyridine	No
Theelol (Doisy):	273.8	+61° (Dline) alcohol	Yes
Tri-acetate	126	-	No
Mono-methyl ether	154.8	-	No

Short stem Anschütz thermometer.

(3) The General Relationship between the Two Forms of Oestrin.

When Marrian (1930) first isolated the tri-hydroxy form he did not regard any of the compounds at that time as the pure hormone, but held that they might in spite of their constant potency and chemical characteristics on repeated crystallization, be only the carriers for the true hormone. Marrian also felt that his compound might be an artefact and be formed from the hydroxy-keto form during his process. He suggests the boiling with KOH as the point of conversion. Doisy and co-workers (1931) while regarding 'theelin' as a pure compound and also regarding 'theelol' as such, feel that Marrian's product (1930) is a mixture of the two forms. This they base on the view that his method would not be expected to separate the two. This, they feel they have done in the preparation of 'theelol' by extraction of most of the 'theelin' from aqueous alkali by ether and the removal of the remaining 'theelin by recrystallization from alcohol. They also feel that the melting point of Marrian's compound 264-266°C. (uncorrected) lies between that of 'theelin' (249°C) and 'theelol' (274°C.) (uncorrected).

Butenandt (1931)[ab] has made a most careful study of the two forms and their relation to each other. He was able to prove that neither compound was converted into the other by the process of extraction so that both existed as such in the original urine. He did this by subjecting the keto-hydroxy form to the prolonged boiling with alkali which Marrian suggested as the point of change.

There was no effect on the chemical or physiological properties of the substance. He subjected the tri-hydroxy form to high vacuum distillation, his method for obtaining the keto-hydroxy form; using both the pure crystals and these mixed with the impure inactive oil which accompanies the substance when it is purified by this means, he showed that no conversion of the keto-hydroxy form occurred. Marrian's substance is left behind in the Butenandt method in the 70 per cent alcohol whereas the keto-hydroxy form passes wholly into the benzene with which the alcohol is extracted. He was able to prepare Marrian's substance from the 70 per cent alcohol fraction of his method. In Marrian's method the keto-hydroxy form is insoluble in 50 per cent alcohol and is left behind in the residue. Butenandt prepared the keto-hydroxy form from this residue. He notes that the amount of the tri-hydroxy form in urine varies greatly. Sometimes half the potency remains in the 70 per cent alcohol in his method and sometimes there is no potency there at all.

Marrian had suggested that his product could not be distilled in vacuo. This was proved to be erroneous. Butenandt showed that the tri-hydroxy form, which was relatively less active physiologically than the keto-hydroxy form, could be converted into the latter by heating in high vacuum with potassium acid sulphate with consequent gain in activity. The distillation product was chemically or physiologically identical with the keto-hydroxy form. This he regards as indicating that the crystals are

the hormone itself and that the latter is not a substance adsorbed on the crystals. The hydrolysis of the acetate of the keto form which is less potent gives the pure keto-form itself again with gain of potency.

Butenandt was able to prepare a product melting at 276°C. which is quite comparable (as melting points go) with that of Doisy. This substance gave no lowering of melting point when mixed with Marrian's preparation which Butenandt says melts at 268°C. (uncorrected). The optical rotation of the two preparations was identical. The physiological potency was, however, quite different. (See the sections on physiological properties and assay) Butenandt suggests, however, that the lower melting point compound is slightly contaminated with the keto-form or that there are two isomeric forms of the tri-hydroxy substance, so that Doisy's suggestion that Marrian's product and his own were isomers of the same substance still remains. However, the optical activities of Marrian's later products at least are very like those of Doisy. Marrian and Haslewood (1932) obtain a rotation of $\alpha 5461 = +76^{\circ}$. Doisy using sodium light got $\alpha D = +61^{\circ}$. Both use alcohol as a solvent.

Marrian (1931) has very recently brought forward evidence that there are two forms of the keto-hydroxy form itself. This is based wholly upon physiological differences since the two forms have the same melting point and optical rotation. He calls these forms A and B. A is the more active form and is obtained

when high vacuum distillation is used in the preparation of the compound. It is relatively unstable, changing into the less active form B merely on standing in alcohol or other solvents. The chemical properties of the two forms are identical as far as has been determined. He believes that Doisy's product is mostly B while that of Butenandt is mostly A.

The significance of this apparent multiplicity of forms of the keto-hydroxy as found by Marrian and the tri-hydroxy as found by Butenandt will be discussed more fully under the section on physiology of the hormones. It is felt that the explanation of much of the confusion existing as to the potency of the hormone lies in the existence of a number of these forms.

The Assay of the Female Sex Hormone.

The assay of the female sex hormone is one of the most important as well as the most confused part of the investigation of this subject. The discovery of pure crystalline substances far from producing a uniform standard has been the source of further confusion. Each laboratory has its own method of

standardization and since even minor variations in technique make a large difference in the result obtained, comparison of potencies obtained in different laboratories is very difficult. Important differences in compounds have been wholly overlooked because of the method of assay.

Methods of Assay.

(A) Chemical.- No really satisfactory chemical methods of assay are known. Kober (1931) has developed a method based on a colour reaction given by the hormone with a mixture of sulphuric acid and phenolsulphonic acid (o + p) and water. Cresol red is used as a standard for the red colour which results. The colour has a green fluorescence. He claims this to be a specific and to agree with biological assay.

(B) Biological Assay.- This is well discussed by Munch (1931). A short summary of methods will be given. The use of the vaginal smear technique in female rodents as a method will be fully discussed.

Estes and Burge (1928) using dried ovaries employed the increased rate of metabolism of paramecia for the detection of the hormone. Frank (1929) showed that the hormone was inert in this respect.

Laqueur found an increased rate of growth of tadpoles when placed in or fed the crude hormone. This was found to be

non-specific.

The growth of the castrate uterus was used by many early workers and also by Frank (1915). He feels that the method is only partly reliable. The comparison of wet or dry uterine weights to body weight is not reliable because of great variability in the latter. He later used the growth in weight of the immature rabbit uterus as a means of assay.

The external signs of oestrus in the bitch and ferret and the production of menstruation are not suitable tests for assay. It is however of importance to know how much of the hormone in terms of units otherwise determined is necessary to give these effects. The changes in the mammary glands of various animals especially the opossum (Hartman, 1926) and Macacus (Allen, 1926) have been used for the detection of the hormone.

Kelly and Papanicolaou (1925) propose the opening of the vagina of the spayed guinea pig, which as mentioned above remains closed except at oestrus, as a test for the hormone.

The albino rat and mouse are the animals most used for the quantitative determination of the oestrus hormone.

Zondek and Aschheim (1926) used the increased growth of the uterus in immature mice. They injected for fourteen days. This is too slow a method and involves the sacrifice of the animals. The increased size of the uterus of the castrate female rat or mouse may also be used as a means of detecting the presence of activity. This has the disadvantage of the necessity of sacrificing

the animal. It is however a good means of checking the vaginal reaction since the latter is not absolutely specific (See below). This is especially important where unusual sources of the hormone such as plants are being tested.

The activity of the excised rat uterus has been used by Frank, Bonham and Gustavson (1925). The rate of contraction of the excised organ in a castrate is one in 65 seconds on an average, when injected and brought to full oestrus it was 1/100-125 seconds. It is not absolutely specific and again necessitates that the animal be killed for each determination. The use of a large number of animals is thus precluded.

Blotevogel, Dohrn and Poll (1926) have described a method for which they claim great accuracy. Blotevogel discovered that the number of cells in the para-cervical ganglion of the mouse uterus which reacted to chromic acid stain (chromophile cells) varied according to the stage of the cycle. They regard this as being due to changes in the amount of adrenalin secreted. The reaction is said to depend upon that of adrenalin. The percentage of cells showing the reaction steadily increases up to oestrus and declines after it. It increases to a much greater extent in pregnancy (from two and a half per cent of the total number of cells in the resting state to 15.5 per cent in pregnancy). This test requires a number of animals and a long time for its performance. It also requires histological technique and is not therefore suitable for a routine method of assay.

The Vaginal Smear in the Female Rodent as a Method of Assay.

This is the most used method of assay of the female sex hormone at the present time. It consists in general in the production of the oestrus reaction mentioned in the section on types of oestrous cycle as occurring in the rat and mouse and guinea pig in an ovariectomized animal by the injection of the extract. There are a large number of variable factors which affect the accuracy of this response. These will be discussed under the following heads:

1. Variations in the Condition of the Animal.
2. Variations in Individual Susceptibility.
3. Variations in Method of Administration.
4. Variations in Criterion of Positivity.
5. Differences between Mouse and Rat.
6. Variations in the Sex Hormones.

(1) Variations in the Condition of the Animal.-

i. General Condition: (a) Age, (b) Nutrition, (c) Infection, (d) Weight.

(a) Age.- The age of the animal at castration provided that it is not before puberty or when the rat is approaching

senility appears to have little effect on the response of the animal. The usual age for castration is about 3-4 months. Kahnt and Doisy (1928) recommend the use of the vaginal smear in the animals to be operated upon for two weeks before the operation, and propose to discard any rats which do not show a normal oestrous cycle. However Coward and Burn (1927) found no difference in response between rats which showed cycles before ovariectomy and those which did not.

(b) Nutrition.- Vitamin B deficiency causes cessation of the oestrous cycle and hence probably would affect the response to oestrin making the animal less sensitive. More important than this Evans (1928) showed that vitamin A deficiency tended to cause continuous cornification in the vagina of the rat irrespective of the stage of the oestrous cycle. This occurring in a castrate would give a false positive reaction.

(c) Infection.- Rats infected in any way before operation should not be used. Allan, Dickens and Dodds (1930) emphasize the importance of careful asepsis in performing the ovariectomy. They state that even if the skin wound heals by first intention an infection may form at the site of ligation of the uterus. This infection may affect the result in two ways: first, by causing a steady flow of leucocytes to appear in the vagina, thus obscuring a positive reaction and secondly, they say that in two cases the presence of a pyometra has been accompanied

by a nearly positive smear in an untreated castrate rat. This would therefore give a false positive reaction.

(d) Weight.- Originally Allen and Doisy (1923) used rats of 140 $\pm$ 20 grams and defined the unit as the amount of material just necessary to produce cornification in a rat of this weight. Bugbee and Simond (1926) have tried to work out a formula for the elimination of the weight effect. Their formula is R.U. = $\frac{W}{140Q}$ where W = weight of rat in grams, and Q represents the amount of hormone necessary for a positive response in the rat used.

Coward and Burn (1927) found that weight had no relation to the size of dose required for a positive response and that they could not diminish the individual variation of rats by taking the weight difference into account.

ii. Operation: (a) Asepsis, (b) Technique, (c) Accessory tissue, (d) Regeneration, (e) Time after Castration.

(a) Asepsis.- The importance of this has been mentioned above.

(b) Technique. The usual procedure is the use of two lumbar incisions. Most workers remove the fat pad round the ovary, the Fallopian tube and part of the uterus together with the ovary itself. Ether anaesthesia is usually employed.

(c) Accessory Tissue.- This is very rare in the mouse

(Parkes, 1929). Most cases which have occurred have been examples of the occurrence of an accessory ovary within the capsule of the normal one.

(d) Regeneration.- Davenport (1925) has reported 83 per cent regeneration when the ovary alone is removed from mice, and 17 per cent when the ovary capsule and tube are removed as well. The regeneration may take place within a month or two of operation but the number of regenerated animals increases up to the sixth month. Reformation of ovarian tissue is not affected by adhesions and minor infections. His proportion of regenerations seems somewhat high. Parkes (1927) states that he found it in 6 per cent of a large series of mice. Hanson and Heys (1927) found 5 per cent showing regeneration in rats. Allan, Dickens and Dodds (1930) found that in 1000 ovariectomies on rats only 0.3 per cent showed regeneration. The incidence of regeneration is thus higher in the mouse than in the rat. In any case the completeness of removal of the ovary at the time of operation should be checked. Kahnt and Doisy (1928) propose to do this by reading the vaginal smear of the operated rats daily for two weeks and rejecting those which show positive smears.

(e) Time after Castration.- Allen and co-workers (1924) found that there was no decrease in sensitivity of the animals up to fifteen weeks after operation. The decrease in sensitivity might be expected to occur on account of the castration atrophy of the organs which takes place after the operation. Allan,

Dickens and Dodds (1930) found that old rats in ill health were much less sensitive than young healthy animals.

(2) Variations in Individual Sensitivity.- The original method of assay (Allen and Doisy, 1923) employed the method of diminishing dosage. That is to say, they found the lowest dose which would bring a single rat of weight 140 ± 20 grams into oestrus. Coward and Burn (1927) showed that the assumption implicit in this method that all rats of approximately the same weight would become positive on the same dose is entirely unjustified. They found that the variation in rats and mice might be as great as 1000 per cent. They believed that not only did rats differ from one another but that the same rat might vary in sensitivity from week to week. They then applied the principles worked out for other biological assays by Trevan (1927) to the assay of the female sex hormone. This method involves the use of a large number of animals on the same dose. A standard curve of response is first established. This is done by the use of at least 100 animals. These are all injected with the same dose of a standard preparation. The number of positives is noted and expressed as a percentage response. Another dose of different strength is given after an interval to the same animals. The percentage response is again noted. This is repeated 3 or 4 times and a curve is constructed plotting percentage response against dosage. Once the curve for the particular colony of rats is established it is

easy to calculate the strength of any preparation. The unit is defined as the amount required to bring 50 per cent of a number of rats injected with the same dose into oestrus. The minimum number of rats given by Coward and Burn is 20 with an ideal number of 100. Allan, Dickens, Dodds and Howitt (1928) regard 20 as too small a number to offset the effects of individual variation. If any preparation gives other than a 50 per cent response as must frequently occur, then the amount of the extract which would produce 50 per cent response can be read off from the standard graph. Coward and Burn also found that there was no difference between the mouse and rat unit calculated in this way. D'Amour and Gustavson (1930) confirmed Coward and Burn's results in the main but claimed that 20 was too small a number of rats to use. They also said that if over 80 per cent or less than 20 per cent of the rats responded, the curve could not be applied accurately. Marrian and Parkes (1929) have calculated the probable error of the results at various parts of the Coward and Burn curve. They find that the probable error is least at the point in the curve just below 50 per cent response. They believe that any part of the curve below 70 percent may be used. According to their results the errors in the higher parts of the curve are greater than in the case of low per cent responses. Laqueur (1926) before Coward and Burn's work recommended the use of at least 12, preferably 24 castrates which have had negative smears for four weeks after castration. He requires a 75 per cent positive response for a unit.

Frank (1929) criticizes the use of large numbers of animals and believes that with care in technique the variations can be reduced. He feels that Laqueur's method mentioned above is the most suitable one.

In an attempt to reduce the effects of individual variation Kahnt and Doisy (1928) have adopted a rather complicated method of selection of animals and of standardizing the individual animal against a known preparation. If Coward and Burn's finding that individual rats vary in their sensitivity from week to week is correct, this standardization of animals is of less value than appears at first sight. Marrian and Parkes (1929) found that there was a surprising constancy in the response of the one group of animals to one mouse unit subcutaneously at different times. It is however of value in rejecting the extremes of variation. Their method of selection is as follows. Daily smears are made for 2 or 3 weeks before ovariectomy, only animals with normal cycles to be selected for operation. Daily smears are also made for two weeks after operation discarding any animals which show positive smears later than two days after ovariectomy. They employ a priming dose; that is, they give 2 rat units to all rats so as to make sure that they all respond; one week after this they inject 1.3 units. If the smears are negative the animals are discarded. One week later test with 0.7 units. Any rats showing positive smears are discarded. Use each animal for no longer than 4 months. Prime all animals if negative on the previous test.

Use a sufficient number of animals. If 75 per cent react in a positive manner, the amount injected is one rat unit. This method is very tedious for routine assay and has some serious faults when used on crystalline preparations will be discussed later under the section on experimental work.

To illustrate the variation in susceptibility of animals, the following result may be quoted from Coward and Burn. The material was injected in oil in a single dose intraperitoneally into rats. This preparation was not crystalline. The number of positives with accompanying doses are as follows:

2.5 mg.-7%; 5.0 mg.-32%; 7.5 mg.-32%;
10 mg.-40%; 12.5 mg.-55%; 17.5 mg.-76%.

Colony Differences.- A uniform stock of rats is essential in order to obtain uniform results. The possibility that differences of assay in different laboratories is due in part to colony differences is reasonable in view of the variation in the age of puberty found in different laboratories and mentioned above. In the case of other animals and other drugs and hormones this type of effect has been shown to be considerable.

(3) Variations in the Method of Administration.-

(a) Route.

(b) Number of Injections, Medium Used,
Time Interval between Injections.

(c) Volume of Fluid Injected.

(d) Time Interval between Tests.

(a) Route.- There are three common routes of administration,- subcutaneous injection, intraperitoneal injection and oral administration. The administration by vagina has also been used especially in monkeys. Since purification has made it possible, intravenous injection has also been tried. Voss and Loewe (1931) using mice found no difference in effect on injecting subcutaneously and intravenously.

Subcutaneous injection is the method most commonly used. Evans and Burr (1926) have shown that intraperitoneal injection is less effective than subcutaneous. It has also been found that intraperitoneal injections do not give more uniform results than subcutaneous ones. Coward and Burn (1927) on the other hand found that there was no difference in the intraperitoneal and subcutaneous dose and claim that Evans and Burr's results were due to individual variations as they used only a few animals.

(b) Number of Injections, Medium Used, and Time Interval between Injections.- The original method was by a single injection. Allen and Doisy (1923) introduced the method of three injections

over 12 hours. Coward and Burn (1927) found no difference between the effect of the same amount of material given in one injection or in three injections at intervals of four hours. However, Laqueur and co-workers (1925) using his water-soluble material emphasized the importance of divided dosage. He gives six injections at intervals over 48 hours. Allan et al (1928) showed that in the case of a water-soluble preparation division of the dose into parts had a great effect in enhancing its potency. For example, when a certain dose given in one injection gave no response on 7 rats, the same dose divided into 6 injections over 48 hours gave a 60 per cent response on 10 rats. Not only the number of injections but their spacing are important. Thus 6 doses over 48 hours gave a response of 55 per cent whereas the same dose given in 4 doses over 12 hours gave no response on 20 animals. Marrian and Parkes (1929) have shown that 4 injections in 36 hours give quite a satisfactory response with water as the medium. Allan, Dickens and Dodds (1930) have shown that 6 doses over 48 hours gives approximately a 25 per cent greater response than 4 doses over 36 hours. The spacing of the dosage over 48 hours may be criticized on the ground that some of the animals are beginning to respond at the time of the last dose or slightly before it so that for them the total amount necessary for a positive response is less than the amount injected. They also show that if oil be mixed with the injection, only 8 units is required for a 50 per cent response whereas when the aqueous solution alone is given 40 units were

required for a 50 per cent response. The unit was based on the amount required when injected in 6 doses over 48 hours. They indicate that the slope of the curve is much steeper with the divided dose than with the single one; that is, doubling the dose will increase the response all the way from no response to 80 per cent whereas 40 times the dose is required to raise the effect of a single injection from no response to 50 per cent response. The slope of the curve for the emulsified solution is intermediate between these two. An advantage therefore of the method giving the steeper curve is that it tends to minimize the individual variation of the rats. Burn (1929) also finds that the divided dose is more efficacious in the case of watery extracts. Kahnt and Doisy (1928) use three injections over 12 hours for both aqueous and oily injections and while admitting that a further division of the dose reduces the weight of the unit, believe that their method gives quite uniform results. Dodds and his co-workers have pointed out that a considerable saving of material is made when the dose is divided. This is a consideration when only small amounts of crystalline material are available. The amount of work entailed in an assay increases as the number of injections increases and has to be balanced against increased accuracy and saving of material in working out a routine method of assay. The decreased individual variation in animals observed with divided doses of aqueous preparations suggest that some of the variation found by Coward and Burn was due to irregular absorption of their oily

preparations especially as intraperitoneal injection has been shown to be less regular in its effects than subcutaneous injection.

The reason for the increased potency when divided doses are used is not known. Most authors suggest that the rate of absorption and of destruction or excretion is the explanation. On this view a single injection of a water soluble material is quickly absorbed and as quickly excreted or destroyed. The division of the dose enables the concentration of the hormone in the blood to be kept up over a longer period of time and hence to affect the sexual organs more efficiently. Marrian and Parkes (1929) say that in the normal rat the hormone acts for at least 24 hours before changes appear in the vagina. The oily solution is regarded as being absorbed more slowly and so producing the result of a continuous injection. McClendon, Burr and Conklin (1928) say that menformone injected subcutaneously into women is excreted in 6 hours. They give no details however and the presence of oestrin in the urine of normal women makes their results doubtful. Fee, Marrian and Parkes (1929) used the heart-lung-kidney preparation. Oestrin was added to the circulating blood and the urine was collected and analyzed for the presence of the hormone. They concluded that small amounts of oestrin appeared in the urine soon after injection into the blood but that only 1 per cent was recovered in this way. The tissues of the heart and lungs did not retain the hormone but it disappeared from the blood almost immediately. The response was

negative in the blood 25 minutes after 250 mouse units had been added to the circulation. Blood when incubated with oestrin in vitro did not cause any destruction after 3 hours at 37°C. They conclude that the hormone is destroyed by oxidation as the blood passes through the lungs. It is questionable whether their results on excretion can be applied to the intact animal.

(c) Volume of Fluid Injected.- Marrian and Parkes found that there was little change in response on increasing the volume of injection from 0.1 cc. to 0.4 cc. They worked with large numbers of animals and used aqueous solutions. Presumably the volume of oily solution injected would have an influence on the response due to the slower absorption of the larger volume. Allan, Dickens and Dodds point out that while the volume does not change the response to a given amount of hormone, yet a volume of 0.5 cc. can be injected more accurately than a volume of 0.2 cc.

(d) Time Interval between Tests.- Kahnt and Doisy (1928) regard this as important. They inject once a week and if two weeks have elapsed since the last positive response they use a 'priming dose' to bring the animals into oestrus and then use them for assay the next week. This is presumably to prevent any marked atrophic changes in the sex organs. Marrian and Parkes (1929) found no regular change of sensitivity, the

extremes being from 40-57 per cent response to a 'unit' of oestrin when the time since ovariectomy of the animals varied from eight to fourteen weeks. They do not say however whether these rats had been used for assay in the interval elapsing since the operation. Kahnt and Doisy find that after being used weekly for about 5 months the animals show an increased, not a decreased sensitivity to oestrin. They propose to discard animals after 4 months for this reason. The reason for the increased sensitivity is not given.

Oral Administration.- This has not been used as a method of assay until recently. The early workers regarded the hormone as being completely inactive by mouth. Laqueur put the ratio oral to injection at 100:1. Allan, Dickens and Dodds by administrating 12 doses by stomach tube found that it required 20 units to bring 25 per cent of animals into oestrus. Loewe finds the ratio to be 20:1. Zondek puts it at 5:1 and Schoeller, Dohrn and Hohlweg (1931) have reduced it to 2.5:1. They used a stomach tube and gave 6 doses. They believe that the purer a preparation is the less is its effectiveness by mouth. This they attribute to accessory substances which promote the absorption of the hormone from the alimentary tract. A solution of the hormone in alcohol of almost any strength is more readily absorbed than an aqueous or oily solution. Allan, Dickens and Dodds regard the inactivity by mouth as due to lack of absorption since it has been shown that the hormone is resistant to digestive enzymes. In contrast to these

results it was shown by Collip (1930)[a] that the alcohol soluble hormone from human placenta was effective by mouth in doses as small if not smaller than those required by injection, when given to the immature rat. The method of feeding was to place the dose in the drinking tube with just sufficient water so that all was taken in 24 hours. This results in an almost continuous absorption of the hormone over this time.

(4) The Criterion of Positivity and the Technique of the Vaginal Smear.

(a) Method of Taking and Reading the Smear.

(b) Requirements for a Positive Response.

(c) Time of Reading.

(a) Method of Taking the Smear.- This varies considerably. Long and Evans (1922) used a spatula and transferred the contents to a drop of saline on a microscopic slide. Other workers use a platinum loop or a cotton plug to obtain the smear. Frank (1929) recommends the use of sterile saline and a pipette. Most workers have found that taking the smear has no influence on the normal oestrous cycle. It does not induce pseudo-pregnancy as does cervical stimulation. The use of too violent a technique in the taking of the smear might stimulate the cervix sufficiently to cause the pseudo-pregnant response. This of course is not important in the case of castrate rats but where the effect of injection of the hormone on non-

ovariectomized animals is being studied, it is a possible factor. Frank (1929), Allan, Dickens and Dodds (1930), stain the smears with haematoxylin and eosin, Marrian and Parkes (1929) with 1 per cent aqueous methylene blue. The first emphasizes the importance of using a different pipette or of careful cleansing between the individual smears on account of the danger of transferring cells from one smear to another. It may be also noted here that the cleansing of the slides is important since a smear may remain partly adherent to the slide and confuse the reading of the next smear. Frank also emphasizes the importance of examination of all parts of the field and of a thorough mixing of the smear. There is some danger of infection of the vagina by the use of dirty pipettes; protozoan infection is also likely to occur. The effect of this on the response is not known. In the case of young rats when the vagina is first artificially opened one is very likely to obtain cornified cells in the smear derived from the plug of cells closing the vagina. These are easily distinguishable however from the true squamous smear. For this reason it is well to take the first smear in the immature animal some time before the full positive response is expected.

(b) Requirements for a Positive Response.- These vary considerably in different laboratories. Loewe (1926) uses the percentage of leucocytes as compared to nucleated and non-nucleated cells as an intermediate reaction. This is not accurate as such mixed smears are difficult to

distinguish from similar ones occurring spontaneously in spayed rats. Laqueur (1928) requires the reduction of leucocytes to a very few with at least equal presence of nucleated and non-nucleated epithelial cells. In his early work Laqueur accepted the smear consisting wholly of nucleated epithelium with a very few leucocytes (the pro-oestrus smear) as a positive result. Allen and Doisy (1923) require full cornification but Kahnt and Doisy (1928) have a criterion very like Laqueur's. Frank and Goldberger (1926) have a scale from 0-4. No leucocytes and nucleated epithelium is 3 and full cornification is 4. Both these are taken as positive. Marrian and Parkes (1929) take full cornification or a mixture of cornified and epithelial cells, but insist on the absence of leucocytes and the presence of cornified cells. Allan, Dickens and Dodds (1930) say that with young and little used rate the smear comes sharply to the end point of full cornification but later after use they tend to show considerable mucus and a clear type of smear is not obtained. They take as positive, smears containing as well as squamous cells a very few leucocytes and a few nucleated epithelial cells. The use of the prooestrus smear (that is, one with nucleated epithelium only) as a positive result has been criticized as this type of smear, since it may contain a very few leucocytes, is regarded as being liable to confusion with variations in the smear of the untreated spayed animal. Allen and Doisy especially deprecate its use in detecting the presence of the hormone qualitatively in new sources. Much of

the work on oestrin occurrence in unusual places mentioned above is open to this objection.

(c) Time of Reading.- The number of rats required to give a positive response before a dose is considered as containing a unit varies in different laboratories. 50 per cent, after Coward and Burn, is the most common requirement. Kahnt and Doisy and Laqueur use 75 per cent. The positive reaction usually appears 48-72 hours after the first injection. If a single injection is given it tends to appear earlier than if the same dose be divided. Frank (1929) believes that if a much larger dose be given than the unit the reaction will become positive in 18 hours and continue for several days. Other workers find that the latent time is not affected by size of dose but that the response with large doses does tend to be prolonged. If the dose is just sufficient to cause a response, the duration of the response may be a matter of a few hours and may easily be missed unless the smears be read frequently. Marrian and Parkes (1929) read smears at 9 a.m., 4 p.m. and 11 p.m. starting 48 hours after the first injection and ending 48 hours after the last injection.

(5) Differences between Mouse and Rat.- Allen and Doisy (1923) state that the dose required for the rat is 2-4 times that required for the mouse. Bugbee and Simond (1926) confirm this and put the ratio at from

2-8 times more for the rat. Coward and Burn (1927) however, found no difference between the mouse and the rat unit. This has been confirmed by Becker et al (1931) using groups of 100 animals. They read the vaginal smears only once 48 hours after a single injection in oil of a crystalline preparation of the keto-hydroxy form prepared by high vacuum distillation. Kochmann (1930) finds the mouse and rat unit for watery preparations 1:4, for oily preparations 1:2.

(6) Variations in the Sex Hormones.- All the above work with the exception of Becker et al, has been done on impure preparations. The isolation of at least two crystalline hormones having different physiological properties and the suggestion that even these may exist in various forms, makes it difficult to correlate the various results of assay. Some of the discrepancies may have been due to different proportions of these compounds present in the impure preparations according to the method used in obtaining them. This will be discussed more fully later.

(7) The Use of Immature Rats.- It has long been known (Allen and Doisy, 1924) that injection of the female sex hormone would cause the opening of the vagina of the immature rat at an age long before normal puberty. This opening of the vagina is also usually accompanied by a positive vaginal

smear. The use of immature animals has been objected to on the grounds of the danger of the occurrence of spontaneous oestrus. If the animals are carefully selected as to age and the age of spontaneous puberty for the colony is known, it becomes safe to use this type of test object. It is the use of the immature animal that has revealed many of the differences between crystalline preparations which are reported in the experimental part of this thesis. Collip (1930) first used the immature animal as a test object on a large scale in the assay of 'emmenin'.

Curtis and Doisy (1931) have used the time of opening of the vagina as a criterion of activity in testing the effect of 'theelin' and 'theelol' on this type of animal. They inject the animals once a day for 3 days since they find that the results when they employ the method described by Kahnt and Doisy (1928) of 3 injections in 12 hours are irregular. Frank (1929) says that the opening of the vagina in the rat is not a satisfactory criterion of the presence of the ovarian hormone since animals castrated at 14 days showed opening of the vagina at the same time as their normal litter-mate controls.

A summary of the method of assay used by various authors is given in Table VIII.

TABLE VIII.

Table of Assay Methods.

Author	Animal	No. of Animals	Medium	Site	No. of Injections	No. of Readings	Time Interval	Per cent Response for Unit
Allen and Doisy (1923)	Rat	1	Oil	s.c.	3	-	12	-
Coward and Burn (1927)	Rat Mouse	20-100	Oil or	s.c. or i.p.	1	2	-	50
Laqueur #(1926)	Mouse	12-24	Water	s.c.	6	6	48	75
Marrian and Parkes (1929)#	Mouse	20	Water	s.c.	4	6	36	50
Kahnt and Doisy #(1928)	Rat	?	Water	s.c.	3	?	12	75
Curtis and Doisy (1931)#	Rat	20	Water	s.c.	3	4	12	75
Allan et al. (1930)	Rat	25-100	Water	s.c.	6	7	48	50
Butenandt #(1930)	Mouse	3-8	Oil	s.c.	1	4	-	75-80
(1931)	Mouse	12	Water or Oil	s.c.	1. 4. 6.	4	-	75
Gustavson #(1930)	Rat Mouse	100	Oil	s.c.	1	1	-	50

s.c. = subcutaneous
i.p. = intraperitoneal
# indicates that the method has been used in the assay of crystalline preparations.

The Physiological Effects of the Oestrogenic Hormones.

A short summary of the general effects of the female sex hormone will be given. Much of this work has been done on impure preparations and is open to some objection on account of the recent discovery of several forms of the oestrogenic hormone, provided that one does not regard the various forms as artefacts due to purification. The influence of impurities on rate of absorption is of great importance in view of the effect of prolonged slow absorption in increasing the effectiveness of a given amount of hormone. The magnitude of this effect is of course quite unknown for the impure preparations used before the isolation of the crystalline substances.

General Actions.

(1) Effect on Circulation and Respiration.- The earliest crude extracts had a depressor action but later workers found little effect on the blood pressure or heart rate. Melchionna (1931) showed that 250 rat units of 'theelol' had no effect on the blood pressure, heart rate and respiratory rate of anaesthetized dogs. Laqueur and co-workers (1926) showed that 2000 mouse units of 'menformone' had no

effect on these functions in the intact animal. D'Amour, Gustavson and Carlson (1931) found that prolonged administration of oestrin to normal female puppies caused no change in arteries, heart, spleen, thymus, liver or adrenals. They gave the oestrin over a period of 17 weeks in a dosage of 400 rat units daily.

(2) Effect on Metabolism.- Laqueur, Hart and de Jongh (1926)[b] found a slight rise in the basal metabolic rate of ovariectomized females on injection of menformon. No effect was obtained in castrated males. Kunde et al (1931) found no effect on the basal metabolism of the normal or castrate bitch when large quantities of oestrin were injected over long periods of time. Typical effects on the sex organs were obtained with this dosage. Verzar and von Arvay (1931) found that menformone and theelin raised the metabolism of female rats 10-20 per cent even after thyroidectomy. There is no action in the normal or castrate male. The rise is absent after pan-hysterectomy and they regard it as representing work done in uterine hypertrophy. Compare David (1931) on the effect of injections on the oxygen consumption of the uterus.

(3) Effect on Growth and Activity.- Bugbee and Simond (1926)[b] state that there was a decline in growth rate of both normal and ovariectomized animals on injection of oestrin. They gave 4 rat units daily for 24 days.

Brouha and Simonnet (1925) however found no effect on growth of immature females. Spencer, Gustavson and D'Amour (1931) found a slight decreased growth in immature male and female rats on injecting 40 rat units of oestrin every other day. The development of both ovaries and testes was inhibited. Bugbee and Simond (1926)[a] found that injections of the hormone would increase the activity of the spayed rat for as long as it was injected. The changes in activity with oestrous cycle have been mentioned above. These disappear in the castrate animal.

(4) Effect on the Anterior Pituitary.- Fluhmann and Kulchar (1931) found that injections of 5 rat units of the hormone every 3 or 4 days for 90 days had no effect in preventing the appearance of the so-called 'castration cells' in the anterior pituitary. Carroll (1931) found that the exposure of tadpoles to theelin for 5 months caused albinism. This he regards as indicating an antagonism between theelin and the pituitary.

(5) Effect on the Sex Organs.-

(a) On the normal female.- The continued injection of oestrin into the normal female rat or mouse (Brouha and Simonnet, 1925) tends to intensify and prolong the prooestrous and oestrous part of the cycle but the

animals do not usually come into continuous oestrus. Hauptstein (1931) investigated the effect of progynon on the normal female and found that with large doses the follicular apparatus in the ovary underwent degeneration and no more corpora lutea were formed. In small doses oestrin has no effect on the follicular apparatus according to most authors (Parkes, 1929). However Mahnert and Siegmund (1927) found that it tended to inhibit ovulation. The anoestrous animal is brought into oestrus by the hormone. During pseudo-pregnancy a larger dose is necessary to cause oestrous changes. This is always the case when functional corpora lutea are present in the ovary. Kunde, D'Amour, Carlson and Gustavson (1930) found that the injection of 200 units daily into the normal or castrate bitch caused hypertrophy of the external genitalia, haemorrhage from the vagina, hypertrophy of the uterine endometrium and appearance of mating instinct. There was hypertrophy of the mammary glands and in one normal case the appearance of large quantities of milk. D'Amour, Gustavson and Carlson (1931) administered 200 units of oestrin daily to female dogs and reported decreased size of the ovaries, and arrested follicular development. There was hyperplasia of the uterine glands in puppies. The hypophysis in the treated animals was smaller than in the controls; this decrease was mostly in the anterior lobe. Parkes (1929) states that oestrus may be induced by injection of oestrin in rats which are acyclic owing to vitamin B deficiency. Smith (1932) has shown that hypophysectomized rats show no less sensitivity to the injection of

oestrin (amniotin and oestrogen) than do ovariectomized rats. If anything they are slightly more sensitive. He found that the prooestrous smear appeared somewhat earlier in the hypophysectomized rats and that oestrus persisted longer in them. He was unable to induce mating in either hypophysectomized or ovariectomized animals with doses as high as 100 units (compare Marrian and Parkes, 1930). He gave injections over 2 days with 4 injections a day.

(b) On the Pregnant Female.- Brouha and Simonnet (1925) found that extracts of liquor folliculi produced no effect on gestation when administered late in pregnancy. Parkes and Bellerby (1926) reported that small doses of oestrin (1-5 mouse units) given in early pregnancy to white mice would cause interruption of the pregnancy and return of oestrus. In late pregnancy larger doses were needed. Parkes (1930) however finds that large doses of Marrian's (1923) crude preparation up to 260 units given in 12 hours in 13 injections failed to cause abortion. In single injections 300 units also failed to have any effect. 360 units given in 19 injections over 36 hours regularly produced abortion. The oestrin in doses of 200 units or over sensitized the uterus to very small doses of oxytocin which were ineffective by themselves and abortion ensued. Where oestrin alone was administered, if abortion was produced oestrus occurred just before. The results of Marrian and Parkes (1930) on the effect of oestrin on castrate animals should be compared with the

above results of Parkes. The order of dosage for what Marrian and Parkes term a true oestrous response is the same as that necessary to produce abortion.

Levin, Katzman and Doisy (1931) tried the effects of the crystalline hormones theelin and theelol on pregnant rats. The rats were 13-16 days pregnant. Doses up to 600 units of theelin and 150 units of theelol were injected over 1 to 3 days. No regular effect on the pregnancy of the litters was observed. In none of the animals did oestrus appear until after the litters were removed about three weeks post partum. They state that they have not investigated the effect of these substances on early pregnancy.

(c) Effect on the Foetus.- It is thought probable by many authors that the placenta protects the foetus from the influence of oestrin which is present in large amounts in the maternal blood. Most authors have noticed no effect on the foetus of injection of large amounts of oestrin (Parkes, 1929). As mentioned above, an active substance has been found in foetal blood but the foetus itself has been uniformly negative. All these tests have been performed on the adult castrate animal and should be repeated on the immature animal with precautions taken to exclude the anterior pituitary hormone.

(d) Effect on the Lactating Animal.- Parkes and Bellerby (1927) reported that oestrus could be produced in the lactating mouse.

The dosage required varied according to the number of young. With a litter of 3 or 4, 2 or 3 units were required, with one of 7, up to 10-12 units were required. The production of oestrus did not interfere with lactation to any degree.

(e) Effect on the Immature Animal.- Allen and Doisy (1924) observed that the injection of oestrin caused the opening of the vagina of the immature rat and a positive smear with typical uterine distension. This they regarded as premature maturity since it occurred about 20 days before normal maturity. They believed since they could get the effect in immature castrate rats that the follicular hormone, not the interstitial tissue, was responsible for maturity. A good discussion of the meaning of the term precocious sexual maturity is given by Engle (1931). Mirskaia and Crew (1930) consider the appearance of typical mating responses as indicating this state. Long and Evans (1922) consider that the bearing of the first litter represents maturity and that unless this occurs earlier than usual the animal cannot be said to be precociously mature. The injections of oestrin into immature mice cause opening of the vagina and the appearance of cornified cells some time before the normal time. Allen and Doisy (1924) at first claimed that these animals mated earlier than usual but this has not been substantiated. The oestrin does not induce ovulation earlier than normal and so pregnancy cannot occur. Even in the case of pituitary implants Ehrhardt (1928) and Seitz (1929) question the

reality of prematurity since they do not find that treated animals bear young earlier than untreated ones.

Doisy, Curtis and Collier (1931) and Leonard, Meyer and Hisaw (1931) have reported the effect of oestrin on the developing ovary of the immature rat. Doisy et al used theelin and gave 3 units daily for varying periods from the age of 21 days. They also gave a single dose of 20 units to other rats on the 21st day of life. The animals were killed at intervals after this. The normals showed steady increase in growth from the time of injection. This rate of growth increased from the second to the fourth week and maturity took place about four weeks after the beginning of the experiment. The singly injected animals showed a retarded rate of ovarian growth for the first week then recovered and the ovaries grew faster than the controls. They remained,however,about 10 days behind in development. The ovaries of those being continuously injected grew much less than the controls and showed hydropic degeneration of the follicles. Leonard et al used an impure preparation and gave 2 rat units daily beginning at the 28th to 37th day of life. They claim that no changes took place before puberty in the ovary but that after puberty if the injections were continued there were less ova and corpora in the treated than in the control rats. These effects together with the effect of oestrin on the male rat (see below) have been taken to indicate that oestrin exerts an inhibitory action on the anterior pituitary. However, Engle (1931) feels that the maturation of follicles before puberty in the

immature mouse mentioned above indicate a pre-pubertial secretion of oestrin and that these small amounts of oestrin stimulate rather than inhibit the anterior pituitary. Mirskaia and Wiesner (1931) in their demonstration of mucification of the immature mouse vagina regard this phenomenon as being due to small amounts of oestrin secreted before puberty since they have been able to duplicate the effect by the injection of very small doses of oestrin. Collip et al (1931) showed that implantations of about 40 mgm. of immature ovaries caused oestrus in adult castrates. Engle (1931) feels that in addition to the endocrine balance between ovary and pituitary other factors determine the time of maturity normally. He feels that exceptionally good nutrition with abnormally rapid growth rate can cause premature maturity. The continuous administration of oestrin to immature animals causes continuous cornification. Zondek and Aschheim (1926) were able to prolong the cornification for 3 weeks. Collip et al (1931) found that small doses (1-3 units) of 'emmenin' produced appearance of vaginal cornification at irregular intervals. Larger doses (10 units) produced a continuous squamous flush which persisted until the time of normal puberty when the animals became cyclic in spite of continued treatment. The nutritional variations may very well account for the differences in time of maturity found in different colonies of animals.

(f) Effect on the Castrate Animal.- It has been possible to reverse almost all the effects of castration by the use of purified crystalline preparations of oestrin. The adult castrate rat or mouse shows vaginal cornification, typical distension of the uterus, the mating reactions and will accept the male. The weight of material needed to produce these effects will be discussed in a subsequent section. The relative amount necessary for the effect on the vagina and on the other sex organs has been discussed by Marrian and Parkes (1930). Most authors believe that the doses necessary for the uterine effect and for acceptance of the male are of the same order as those needed for the vaginal effect. Marrian and Parkes however conclude that 200-400 times the dose is needed for uterine oestrus and copulation. They were also compelled to inject the amount 19 times in 36 hours to get effects even with this dosage. They used Marrian's preparation (1923) which he later used for the preparation of the tri-hydroxy form of oestrin and which contains little of the keto-hydroxy form. They conclude that the amounts of oestrin present in human pregnancy are not as enormous, physiologically, as they had been considered and that enormous doses are needed for any effect on the human. Other workers using other preparations have found that a few injections of a relatively quite small dose of oestrin will cause uterine effects. David (1931) for example found an increase in the oxygen consumption of the uterus of immature mice with doses of 1-1.5 rat units of an impure

preparation injected 48 hours before killing the animal. He was able to get increased oxygen consumption in adult castrates accompanying vaginal oestrous changes by the use of 10-20 rat units.

(g) Effect on the Senile Animal.- Steinach, Heinlein and Wiesner (1925) report the production of oestrous changes in the senile anoestrous mouse by the injection of oestrin. Later Steinach, Kun and Hohlweg (1928) report the occurrence of ovulation in these animals. Most authors do not believe that oestrin causes ovulation in the animal at any period of life. Butenandt (1931) has also reported temporary rejuvenation by the injection of his impure progynon. Three months' treatment caused considerable rejuvenation but the rat again became senile after the cessation of the injections.

(7) Effect on the Male Animal.- Laqueur, Hart and de Jongh (1926) showed that the injection of menformon caused a repression of the growth of the testis in immature male animals. de Jongh and Laqueur (1930) showed that 50 units of menformon daily administered to male guinea pigs for 4 weeks caused preliminary mammary hypertrophy; if the dose is then suddenly decreased lactation begins within 3 days and continues for as long as 40 days if continuously decreasing doses are given. He compares this to the production of lactation in women post partum where the concentration of the hormone in the blood is also rapidly falling. Wade and Doisy (1931) studied the

effect of theelin and theelol and extracts of liquor folliculi on male rats. They gave 1.25-20 units daily for 19 days to rats of 20-30 days of age. The testis weight relative to body weight was decreased in all cases, the decrease being greater with higher dosage and rather greater with liquor folliculi than for the pure products in corresponding doses. There is no direct antagonism between the male and female hormone since they did not interfere with one another's action when injected simultaneously into birds (Freud, de Jongh and Laqueur, 1930). The cause of the repression of testis development by the female sex hormone is considered by Moore (1931) to be a repressant action of the hormone on the anterior pituitary gland. The significance and effect of the presence of femalehormone in males normally is not known.

(8) Effect on the Plumage of Birds.- Juhn, D'Amour and Gustavson (1930) and Freud, de Jongh and Laqueur (1930), have shown that the injection of pure crystalline oestrin (keto-hydroxy form in the case of the first group of authors) will cause the development of female plumage in the male bird whether castrated or not. The former group of workers found that in the adult capon feathers regenerating from plucked areas are of the female type while the injections are continued. Failure to inject the hormone even for a single day resulted in a band of colour of the male type appearing on the

feather. They also found hypertrophy of the oviduct in pullets.

(9) Effect on Plants.- It has been noted that oestrin stimulates plant growth. This is in accordance with the occurrence of this substance in growing plant tissue.

The Physiological Activity of Crystalline Oestrogenic Hormones.

The unit of an oestrogenic substance has been so variously determined by different authors that it is difficult to compare the results obtained in different laboratories with crystalline preparations and to determine whether the variation in weight of a crystalline substance represents a true difference in physiological potency or merely reflects differences in assay method. Both rats and mice and both mature and immature animals have been used as test objects. It is still uncertain what the relative units for these different test objects are. There are also considerable differences in the time of appearance of oestrus symptoms after the

injection and the length of time over which they persist. Again it is difficult to say whether these are real or assay differences. At present the only satisfactory way to test the relative potencies of the various oestrogenic hormones is for a laboratory to establish its own method of assay, to prepare the various compounds to be tested and to compare them with identically the same assay technique. It was fairly soon established that the tri-hydroxy form was less potent on the adult castrate rat than the keto-hydroxy form. A table adapted from Butenandt and Hildebrandt (1931)b illustrates this difference and the effect of dividing the dose on the unit.

TABLE IX.

Compound	Weight of Substance per Mouse Unit			
	One Dose	2-3 Doses in 12 hours.	4 Doses in 36 hours.	6 Doses in 48 hours.
Butenandt: Keto-hydroxy. (M.P. 250-251°C.)	0.125γ	0.07γ	0.033γ	0.025γ
Marrian: Tri-hydroxy. (M.P. 269°C.)	0.66γ	0.25γ	0.125γ	0.10γ
Butenandt: Tri-hydroxy. (M.P. 276°C.)	5-10γ	-	-	-

The first method is comparable with Gustavson's, the second with Doisy's, the third with Marrian and Parkes's and the fourth with Laqueur's as far as division of the dose is concerned. All injections were made in sesame oil. The value obtained by Butenandt for Marrian's product is in good agreement with that obtained by Marrian himself. Marrian obtains a unit of 0.11-0.14γ giving the dose in 4 parts over 36 hours in water.

For the keto-hydroxy form, it is very difficult to compare Doisy's theelin with the value obtained by Butenandt. Doisy used rats and obtained a unit of 0.38γ for 3 injections in 12 hours. D'Amour and Gustavson (1930) using a single injection in oil also get a unit of 0.38γ though they only require 50 per cent response and Doisy requires 75 per cent. There is probably a balance of differences of assay here. If one regards the mouse and the rat as having the same unit, then Doisy's theelin is about a fifth as active as Butenandt's preparation. If the ratio 4:1 for the rat-mouse ratio is taken, then it has only slightly less activity.

Butenandt holds the view that two forms of oestrin are present as such in pregnancy urine and that they are not artefacts produced in the course of preparation. An indication that the existence of only two distinct chemically pure compounds closely related to each other, each having slightly different but well-characterized physiological potencies,will not account for the facts is seen in the third preparation in the table. Butenandt

modified the method for the tri-hydroxy form and obtained a product which had a melting point of 276°C.; however this was identical with Marrian's product as far as specific rotation was concerned and a mixed melting point of the two preparations caused no alteration in the melting point of the 276° C. compound. Moreover, Marrian's later (1931) preparations give a melting point and rotation closely approximating that of Butenandt's compound. The analysis and molecular weight of these compounds were also identical. In contrast to all this chemical and physical similarity, the physiological potency is seen to be only about a fortieth or a fiftieth as great. It may be mentioned here that a report of the preparation by the present author of a crystalline substance from human placenta having a very low potency on the adult castrate but apparently having the same melting point as Marrian's earlier preparations, was made by Dr. Collip some time before the appearance of Butenandt and Hildebrandt's paper (Collip, 1931). Butenandt was still able to convert this low potency tri-hydroxy form into the active keto-hydroxy form by heating with potassium acid sulphate.

Butenandt suggests as an explanation for this low potency that the tri-hydroxy form is completely inactive on the castrate mouse, any activity which it possesses being due to a mixed crystal formation with the keto-form. He regarded this contamination as being a constant quantity since recrystallization from different solvents did not change the potency of a preparation. His other suggestion is that the inactive form is an isomer of the active

tri-hydroxy form. The type of isomerism is not stated but it does not alter the melting point, mixed melting point, or specific rotation of the compounds. The contamination of the keto-hydroxy form necessary to give a potency of one mouse unit in 10γ would be only one per cent. Butenandt reports that Dohrn and Hohlweg using his method have prepared a low potency crystalline substance from mare's urine.

This suggestion that there were two forms of tri-hydroxy oestrin has recently been followed by a similar suggestion for the keto-hydroxy form. Marrian (1931) pointed out certain discrepancies between the results of Butenandt and of Doisy. Butenandt finds that the keto-hydroxy form is 4 or 5 times as active on castrate mice as is the tri-hydroxy form, this holds fairly closely for whatever the number of divisions of the dose are provided that corresponding degrees of division are compared. He also finds that the keto-form has a longer latent period and the action is more prolonged than the tri-hydroxy form. Doisy on the other hand finds theelin (keto-hydroxy) about twice as active as theelol (tri-hydroxy) and claims that the latter has the more prolonged action. Marrian tested a sample of Butenandt's substance about two weeks after he received it. Butenandt had reported a potency of 0.025γ per mouse unit. Marrian had to use 0.063γ, or more than twice as much. He then obtained a fresh sample from Butenandt and tested it immediately. The weight of a mouse unit was 0.025γ. On keeping for a month in a vacuum dessicator, the potency fell to 0.053γ per unit. There-

after there was no further change in potency after two more months. There was no change in the melting point of the compound as this change in physiological potency took place. Butenandt found that the potency of a preparation of his crystals showed a decrease to one third of their potency on keeping in alcohol for two months. Marrian suggests that this was a change from the active form which he calls A to the relatively inactive form B. He regards Butenandt's compound and presumably all preparations obtained by high vacuum distillation as consisting wholly of the A form when fresh. He considers that Doisy's method results in the B form almost entirely. He bases this on the ratios between the potency of the keto-hydroxy and the tri-hydroxy form. The A form is five times more active than the tri-hydroxy form and this is Butenandt's ratio and the B form is about 2-3 times as active as the more potent tri-hydroxy form as prepared by Marrian and this is Doisy's ratio for theelin and theelol. He believes that the melting point and specific rotation of the two forms are identical since Butenandt and Doisy give the same values for these. A comparison of the melting points of the derivatives of Doisy and Butenandt's product in Table VII will show that these are also nearly alike. Marrian finds a further difference in physiological potency between the A and the B forms of keto-hydroxy oestrin. Giving four doses in 36 hours the latent period and length of persistence of the oestrus differs greatly. The tri-hydroxy form shows the greatest number of animals positive at 24 hours after the

last injection. The number falls off rapidly so that at the end of the next 24 hours all the animals are negative. The B form of keto-hydroxy oestrin shows a slightly longer latent period, the maximum number of positives coming at 34 hours. The effect is slightly more prolonged, up to 72 hours after the last injection. The A form differed greatly from both these forms. It showed a sharp maximum of positives at 48 hours after the last injection and then a second maximum lasting from 108-120 hours after the last injection. He again concludes that Doisy had the B form since he could not possibly have thought that the tri-hydroxy form had a more prolonged action than the A form. Why D'Amour and Gustavson (1930) did not get a higher potency for their preparation which since it was obtained by vacuum distillation, was presumably on Marrian's view the A form is difficult to see. The explanation may lie in the long latent period. They read their rats once only 48 hours after a single injection. It is reasonable to suppose that they might miss a good proportion of late positives in this way. The relative potency of these forms on the rat is not known since the work has all been done on mice. Becker et al (1931) who found an equal unitage for the rat and the mouse also read only once at 48 hours and used D'Amour's preparation. This work should be repeated on the light of Marrian's work.

If Marrian's view is correct it makes the evaluation of past results even more complicated than before. The question as to whether any or all these forms actually exist in the body is also

important. It may be mentioned in connection with the relation of the various forms of keto-hydroxy and tri-hydroxy oestrin. Ludwig and von Ries (1931) found that impure progynon is unaffected by X-ray, is destroyed by ultra-violet light and the potency is doubled by red light. Red light also recovers the hormone potency destroyed by ultra-violet light. It might be expected from Allen's earlier results that light would inactivate the hormone; but the increased activity with red light and the restoration of the potency by ultra-violet light are remarkable. Since the various forms of the active substance are indistinguishable chemically except into the two classes of keto-hydroxy and tri-hydroxy, it is possible that these effects of light represent a conversion of the substance from one form to another. It would be interesting to repeat this work on the pure preparation with possibly a measurement of the absorption spectrum before and after treatment.

Action of Derivatives.- Butenandt (1931)[a] showed that benzoylatin an impure preparation of the hormone had no effect on the weight of the unit. However, the benzoylated hormone had a slightly longer latent period and a much prolonged action. This he regards as being due to slow hydrolysis of the ester in vivo and a consequent slow continuous action of the hormone. He also found that the pure crystalline mono-acetate of the keto-hydroxy form was active in the same dose as the hormone itself and showed a slightly prolonged action. Butenandt also showed that the

hydride was inactive. Marrian (1932) finds that methylation of the phenolic hydroxyl group (formation of a mono-methyl ether) of his tri-hydroxy form inactivates it, whereas esterification of the three hydroxyl groups causes no change in potency.

Action of Crystalline Substances on Immature Animals and by Mouth.

Curtis and Doisy (1931) using the immature rat showed that the amount of theelin necessary to cause the opening of the vagina in three out of five 18 day old rats within ten days of the injection is 1.08γ or three times the adult castrate unit. They injected once a day for three days. Theelin by mouth was equally active on the immature rat as by injection. Theelol required only 0.16γ or one quarter the adult castrate unit to cause similar effects on the immature animal. Theelol required to be given in 1.5-2.0 times the subcutaneous dose when given orally to the immature animal. They also administered theelol orally to spayed adult rats and found the oral unit 2-3 times the subcutaneous unit.

The use of the opening of the vagina as a criterion, the long period after the injection over which its effect is supposed to extend, and the use of such small numbers of animals are points of criticism of the results of these authors with immature rats.

A table of the relative potencies (in terms of number

of γ per unit) of the various crystalline preparations is given below. Some of the conditions under which the potencies were obtained have also been indicated.

TABLE IX.

Physiological Potencies of Crystalline Oestrogenic Substances.

Compound and Author	Potency γ per unit	Animal	No. of Injections	Time Interval	No. of Animals	Percentage response for Unit
Keto-hydroxy form. Theelin (Doisy)	0.38	A.R.	3 (W)	12	20	75
	1.08	I.R.	3 (W)	48	5	60
	1.08	I.R.	Oral(W)	7	5	60
Hormone (Butenandt)	0.03	A.M.	4 (O)	36	10	80
	0.07	A.M.	3 (O)	12	12	80
	0.125	A.M.	1 (O)	-	12	80
Mono-acetate	0.167	A.M.	1 (O)	-	12	80
Hydride	Inactive					
Gustavson & D'Amour	0.38	A.M.	1 (O)		100	50
	0.38	A.R.	1 (O)	-	100	50
Marrian:						
Keto-hydroxy A.	0.025	A.M.	4 (W)	36	20	50
Keto-hydroxy B.	0.060	A.M.	4 (W)	36	20	50
Tri-hydroxy form. Marrian:						
Tri-hydroxy.	0.13	A.M.	4 (W)	36	20	50
Butenandt: M.P.						
Tri-hydroxy(269°C.)	0.68	A.M.	1 (O)	-	12	80
	0.125	A.M.	4 (O)	36	12	80
Tri-hydroxy(276°C.)	5- 10.0	A.M.	1 (O)	-	12	80
Theelol (Doisy)						
(274°C.)	0.68	A.R.	3 (W)	12	20	75
	0.16	I.R.	3 (W)	48	5	60
	0.32	I.R.	Oral(W)	-	5	-
	1.35-2.0	A.R.	Oral(W)	-	?	-
Emmenin Crystals	16.0	A.R.	6 (W)	36	100	60
(274°C.)	1.55	I.R.	6 (W)	36	30	47

A = adult, ovariectomized; I = immature (normal)
R = rat;
W = water medium;
O = oil medium.

E X P E R I M E N T A L W O R K.

Methods used generally throughout the investigation will be described here. Any special methods used for only part of the work will be described as they make their appearance in the investigation. The chronological method will be followed in the description of the experiments. The different types of experiment followed on each other and each new type was suggested by previous work. It is hoped, therefore, that the chronological method of reporting them will make a logical account of the development of this research.

THE METHOD OF ASSAY.

The animals used for assay of the preparations throughout were albino rats. The colony was bred from Wistar stock and has been inbred for about 15 generations in this laboratory. They were

kept in sectional cages for the most part. When the animals were being fed extracts they were transferred to separate box cages.

The diet of the colony was a modification of McCollum's mixture and consisted of rolled oats, corn meal, bone meal, linseed meal, whole wheat flour, powdered whole milk and salt. In addition to this green vegetables and meat were fed once a week. A little yeast powder and wheat germ was given once a week. Most of the animals used for castration were 3-4 months old. Any rat found to be infected (middle ear disease or lung infection) was discarded. No attempt in assaying was made either with the mature or immature rats to correct for weight differences.

Operation.- A. Ovariectomy.

On Adult Animals.- Ether anaesthesia was employed. Alcohol was applied to the area of the incision. The instruments were kept in alcohol and the hands of the operator were well washed and soaked in alcohol before the operation. The incision used was a lateral transverse one, situated in the lumbar region on each side. The ovary and its fat pad were withdrawn from the abdominal cavity. The uterus was ligated about one third of its length from the distal end. The ovary, fat pad, Fallopian tube and part of the uterus were removed. The wound was closed in two layers. The muscle was sutured separately from the skin. It is felt that this helps to prevent infection from spreading in along the sutures and also reduces the chance of the wound breaking down.

The suturing material used in the early part of the work was catgut, that in the later part was black silk. The impression is that infections are less common with the former. The mortality from the operation was practically nil. The occurrence of regeneration has also been rare. The animals have been used at varying times after castration, some the same week, some not for several weeks after. No attempt has been made in most cases to follow the vaginal smear before using the animal. If a positive smear appeared at an unusual time the animal was kept under observation. The animals have been used for about 6-7 months after castration.

On the Immature Animal.- The anaesthetic and preparation were the same as for the adult rat. The incision was made ventrally just above the pubis. The bladder was gently withdrawn and the uterus found at the base of the bladder. In the case of over distended bladders which frequently occur in young rats, care was taken not to rupture the bladder. The horns of the uterus were drawn down and the ovaries removed by pulling on the uterus until the ovary and its fat pad came away. The uterus was cut without ligation. Haemorrhage from the cut uterus was negligible. The presence of the ovary in the fat pad was confirmed; if it was not demonstrated a lateral incision was made and the ovary was usually easily found partly detached from its connections. It was then removed. The double layer of sutures was employed in the case of some of the immature animals but not in all.

B. Transplantation.-

A second type of operation has been performed, that of transplantation. This has been performed for the most part on immature rats, but adult female castrates have also been used. The site of implantation was the spleen in both types of animal. A lateral incision was made just below the ribs on the left side. The spleen was withdrawn from the incision. Meanwhile another operator removed the ovaries from the donor animal. The spleen of the recipient was cut into by means of a tapering knife blade without a handle. An attempt was made to have the opening of the incision smaller than its internal extent so that once the ovaries were placed in the spleen they would be held in place. Surprisingly little bleeding was encountered. The knife blade should be sharp, otherwise the spleen tends to tear. The ovaries, first stripped of their capsules, are transferred to the spleen as soon as they are removed from the donor. In the case of immature ovaries implanted into adult or immature spleen, the whole ovary is used and the pair are placed in the same incision. In the case of adult ovaries implanted into the immature spleen the ovary is first divided and part is placed in the spleen. In most cases the rapid coagulation of the blood retains the ovary and no ligature around the spleen is necessary. Should one be used it is well to tie it only loosely since a tightly tied ligature interferes with the blood supply and with the chance of the transplant taking. The incision was closed in two layers, muscle and skin as before. The mortality from the

operation was very low. Most of the deaths in both this and the castration operations were due to an overdose of anaesthetic. Once a rat has stopped breathing it is very difficult to restore.

The age of the immature animals used both for operation and for assay was usually 21 days. It varied from 20-23 days at the beginning of the assay.

Method of Administration of Extracts.- The medium used was water. In the case of crude preparations they were either dissolved in dilute alkali and carefully just neutralized, or were dissolved in alcohol first and then diluted with water. The dilutions were usually so great that no difficulty in obtaining a uniform solution was encountered. The injections were all made subcutaneously. The needle was passed well into and along the subcutaneous tissue to prevent leakage of the injected material. It was not found necessary to use collodion for this purpose. The volume injected varied. In the immature animal it was usually 0.2-0.3 cc. In the earlier part the number of injections was three at 4 hour intervals. In the later part six injections in 36 hours were given. The spacing of these injections was not even, three were given on two successive days at about 10 A.M., 2 P.M. and 6 P.M. The shortest time between assays was usually one week. At one time the practice of priming was employed.

Oral Administration.- This method was used mostly on the immature animal. They were placed

in box cages which were divided down the centre so that the two animals could be placed (one in each partition). The amount to be fed was placed in the drinking tube. These tubes were made of glass, the open end was drawn down so that the water escaped only when the animal licked the end of the tube. The dose to be given was placed in the tube together with enough water so that the animal drank all the contents over 24 hours. The total dose was usually given over a period of three days. If the animal did not take the whole dose on any day the amount left was measured and discarded and a fresh full day-dose was placed in the tube. An attempt was made to adjust the volume so that none was left and on the other hand that it took about a day for the animal to drink the total amount. The essential feature of this method of feeding was that the dose was administered in a practically continuous manner over a day. The effect was one of great division of the dose. In conformity with this we find that the weight required for a unit is the same or smaller than that required for injection in the case of the immature rat. This is not true for the adult castrate where the oral dose is greater than the injection one. If the rats did not take all the fluid, the amount remaining was measured and the proportion of the dose taken recorded. If on the other hand the tubes were observed to be empty in half a day, water was given before the second dose so that the animal would not drink the whole amount in a short time. The amount of evaporation from the tubes was quite small even on warm days. On the first day of

feeding the animals often did not take the whole amount. They could be taught to drink from the tube and usually took the full amount after the first day. The volume of water used was three and one third cc., thus the total dose was given in 10 cc. over three days. In the case of continuous feeding the volume was raised to 5 cc. and later to 10 cc. daily as required.

Method of Taking and Reading the Vaginal Smears.- The smears were taken by means of a pipette with a rubber bulb so that the contents could be readily aspirated and expelled. The pipettes were well washed between each smear. Isotonic saline (0.9% NaCl) was used. It was not sterile. The pipettes were boiled daily. The saline was injected into the vagina, withdrawn and transferred to a microscopic slide. It was examined fresh without staining, and with the low power of the microscope. In case of doubt acetic acid was added and the nuclei of the cells examined under high power.

In reading the smear the total number of cells was given the arbitrary value of four plus. The proportion of the various types of cells present was indicated by the number of plus signs. Thus a smear with a large number of squamous cells and a few epithelial cells would be designated three plus squamous, one plus epithelial. If the number of any type of cell was very small it was designated as 'occasional'. A smear was taken as indicating a

positive reaction when it consisted wholly of cornified cells or when the proportion of squamous cells was greater than that of nucleated epithelium. The presence of more than a very occasional leucocyte was considered to render the smear negative. This standard is purely arbitrary. As has been mentioned above almost any change from the castrate smear may be taken as indicating some activity in the preparation being assayed. However, for purposes of comparison this standard was adhered to. In order to register the smears of a number of animals which reached the stage of disappearance of leucocytes and consisted wholly of epithelial cells but which never progressed to the fully cornified stage, they have been included with the positive smears and the total response squamous and epithelial types has been designated as 'epithelial' and the percentage of the animals showing this type of response recorded. It is fairly common for immature rats to reach the epithelial stage and remain there for some hours and then regress to the leucocyte stage. It has been found that if the epithelial stage persists for a long time the animal usually does not progress to the cornified stage.

Certain sources of error in reading the smears will be noted here. In the case of the immature rat the plug of cornified cells closing the vagina have to be broken down at the first reading. This often results in their appearance in the smear. They should be distinguished from a truly cornified smear. For this reason it is well to take the first smear before the positive reaction is expected.

There is some difficulty in distinguishing between small rapidly dividing epithelial cells showing mitotic figures, and leucocytes. It has however been found that the animals showing this evidence of rapid division do not often reach the squamous stage. The size of the squamous cells varies greatly and sometimes it is difficult to distinguish small cornified cells from epithelial cells with nuclei. The absence of a nucleus and the rather ragged edge of the squamous cell helps to characterize it.

It is extremely important that the smears should be uniformly mixed on the slide.

The readings were taken 48 hours after the first injection or after the commencement of feeding. Smears were taken at intervals after this until all the animals showed a squamous smear or the appearance of leucocytes thus indicating regression. When an animal became 'positive' the smear was not read further so that in general no data are available as to the duration of the positive response.

The number of times of reading and the interval between them were determined by the reaction of each group of animals. If any showed signs of becoming positive they were read again in a few hours. All animals were read morning and afternoon for two or three days until they became positive or regressed. In the later assays the number of readings was increased and the smears were often read several times at night. It is felt that the chance of missing a positive by this method is relatively slight.

The number of animals used in the first experiments was quite small and the diminishing dose method was employed. As the importance of individual variation was recognized, the number was increased to 20-30 on a single dose in the case of the adult castrates, and to 10-20 per dose in the case of the immature normals and castrates. For the latest assays which were confirmatory in nature, 100 adult castrates were used. The individual variation of most of the animals was fairly small, doubling the dose was usually sufficient to change a poor response into a good one. In the case of the immature animal, the spread was even less. This decrease of individual variation as compared to the 1000 per cent reported as the outside limit by Coward and Burn (1927), is probably due to the division of the dose as mentioned in the discussion on assay.

The methods of preparation of extracts will be discussed in connection with the various extracts made.

The experiments will be described and discussed at the same time. It is felt that this will make for greater clarity than reserving all discussion to a separate section.

THE BEGINNING OF THE INVESTIGATION.

This investigation was started in an endeavour to prepare oestrin from the ether extract of human placenta which was discarded in Dr. Collip's method for preparing emmenin. The oestrin was required for some work on basal metabolism rates on rats. The effect of various sex hormones on the metabolic rate of adult and immature rats was being studied. It was thought desirable to use a pure crystalline product.

Marrian's method of preparation (1930)ab was therefore carried out.

Extract A.

The ether extract used here had been stored in alcohol at room temperature for several months. For the last part of this period it had been placed over the radiator so that the alcohol had evaporated leaving a tarry mass. The preliminary treatment which this material had received in the emmenin process will be summarized. The placenta was ground while fresh and mixed with 1-1/8 volumes of acetone and filtered. The acetone was distilled and the water solution concentrated. The solution was mixed with enough alcohol to bring the percentage concentration to about 80

per cent. The precipitate was removed and the alcohol filtrate concentrated. It was acidified with glacial acetic acid and extracted five times with ether. It was the concentrate from this ether extract which was used as a starting point for the preparation of the crystals. The total weight of placenta which the concentrate represented was 300 kilos. One half of this was taken. It was dissolved in 3 litres of 5 per cent KOH and placed on a boiling water bath for 3 hours. Carbon dioxide gas was passed in for 12 hours. A bulky precipitate appeared. The solution which now showed a pH of about 8 on testing with 'universal indicator' was diluted to 6 litres with distilled water and extracted 12 times with ether. Some difficulty with emulsification was encountered and the material had to be centrifuged in large cups to produce complete separation of the ether. The combined ether extracts were washed four times with 0.2N HCl and twice with water. The ether was then distilled to dryness. The residue, which was dark brown in colour, was dissolved in 200 cc. hot acetone and left in the ice box for several days. An almost white crystalline material separated. This was filtered off. The crystals were soluble in hot acetone but crystallized out in the cold. Upon attempted recrystallization from acetone they separated as a semi-crystalline mass. The precipitate before attempted recrystallization was treated with acetone as above several times to remove traces of the active material. These washings were added to the original acetone filtrate and the acetone was evaporated in vacuo. The residue was dissolved in 100 cc. of

ethyl alcohol (95%) and enough water added to make the alcohol content 50 per cent. A precipitate formed which partly disappeared on boiling and partly appeared as an immiscible oil floating on the surface. The mixture was boiled for 15 minutes, then placed in the ice-box at a temperature of -15°C. over night. The oil turned into a waxy material and the liquid was filtered from it in the refrigerator. The filtration was somewhat slow. The residue on the filter was washed back into the original flask with hot chloroform alcohol mixture. The solvents were removed and the residue again boiled with 50 per cent alcohol, chilled and the solution filtered. This was done four times, the 50 per cent alcohol filtrates combined and evaporated to dryness. The residue was dissolved in a large volume of ether, and the ether extracted with ten 50 cc. lots of KOH. It was noted that with the addition of the first lot of KOH the colour of the ether extracted changed from a pale yellow to a deep reddish-brown and almost all the colour went into the alkaline solution. The writer is informed that this is a reaction characteristic of the flavones. The alkaline solutions were combined and acidified with 110 cc. of concentrated HCl. This caused a disappearance of the red-brown colour and the appearance of a precipitate which tended to dissolve as more HCl was added. The acid solution was extracted eight times with 200 cc. of ether, the ether washed with water and evaporated to dryness. The residue which was a brown oil was dried and then softened with a little alcohol. The substance was placed in a centrifuge tube. 10 cc. of ether

(redistilled) was added and the tube placed outside the window or in the ice box, if the outside temperature was too high, at a temperature of -17°C. for some hours. When the ether was first added a granular precipitate separated. Not much more came out on chilling. The tube was centrifuged rapidly while still cold. The precipitate was washed with cold ether. It was light brown in colour. It was next dissolved in 95 per cent ethyl alcohol and boiled with 'norite' for 30 minutes. The solution was almost colourless. The treatment with charcoal was repeated with the same alcohol solution. The charcoal was washed with hot alcohol twice. An air reflux condenser was used. The centrifuge tube was attached by a cork to the bottom of an ordinary burette. The alcohol was evaporated off. Since it was thought desirable to keep the material in the same centrifuge tube until recrystallization, a method of evaporating the alcohol and ether in the tube itself was used. This merely consisted in placing the tube in a beaker of hot water and blowing air into it. The water should be below the boiling point of the mixture so that it does not tend to leap out of the tube. This does not tend to occur as long as the blast of air is on. The residue in the tube which was yellow-white in colour was dissolved in ethyl acetate and set aside to crystallize in the ice-box. No crystals formed.

This material was assayed on adult castrate rats. The expected units were calculated from the activity present in the original placenta.

The assay was:(6 doses in 48 hours = Total Dose.)

One rat	2/3 unit.	Response - positive
One rat	15 units	Response - positive
One rat	30 units	Response - positive

The material was diluted further and

One rat	4/5 unit	Response - positive
One rat	1/5 unit	Response - negative.

According to this rough assay the potency was much as would be expected from calculation. An assay on 1/25 unit and 1/50 unit were negative.

Some two weeks later, on December 22nd, 1930, a further attempt was made to obtain crystalline material. The ethyl acetate was placed in a small round bottom flask, and an equal volume of distilled water was added. The ethyl acetate was evaporated off in vacuo. As soon as the acetate had been distilled the water phase became opaque. On examining this fluid under the microscope the turbidity was seen to be due to needle-like crystals arranged in rosettes. The water was heated to 70°C. The volume was about 20 cc. Part of the crystals dissolved and part flocculated, descended to the bottom of the tube and was centrifuged off. On cooling the water, crystals separated out. These were centrifuged off and dried in the centrifuge tube in a dessicator over $CaCl_2$. The tube was weighed, the crystals dissolved out with alcohol in which they were readily soluble. The tube was weighed again. The weight of the hot water soluble crystals was 9.5 mg. This indicates a solubility in hot water of 0.05 per cent approximately.

This solubility was much greater than would be expected for the sex hormones which were believed to be almost insoluble in water.

A melting point was done. The apparatus used was a Florence flask with a wide bore test tube in the mouth. The flask and tube were partly filled with paraffin. The long stem thermometer projected above the flask from about the 120°C. mark up. Only about 2" of the thermometer were under the paraffin in the inner tube. The correction for this type of apparatus at 270°C. is about + 9°C. The crystals showed slight darkening at 243°C., further darkening and shrinking at 251°C. and melted at 258°C. (uncorrected). Marrian claimed that his early preparation melted at 256°-261°C. rather indefinitely with decomposition. This seemed to be reasonably good agreement; but the water-solubility was a difficulty as Marrian's preparation would presumably be quite insoluble in neutral solution.

Characteristics of the First Crystalline Preparation from Human Placenta.-

The water-soluble crystals gave the Lipschutz oxycholesterol reaction very faintly. The Liebermann-Burchard reaction was negative both on hot water-soluble and hot water-insoluble crystals.

That part of the crystal fraction which was insoluble in hot water was regarded as a different substance though the

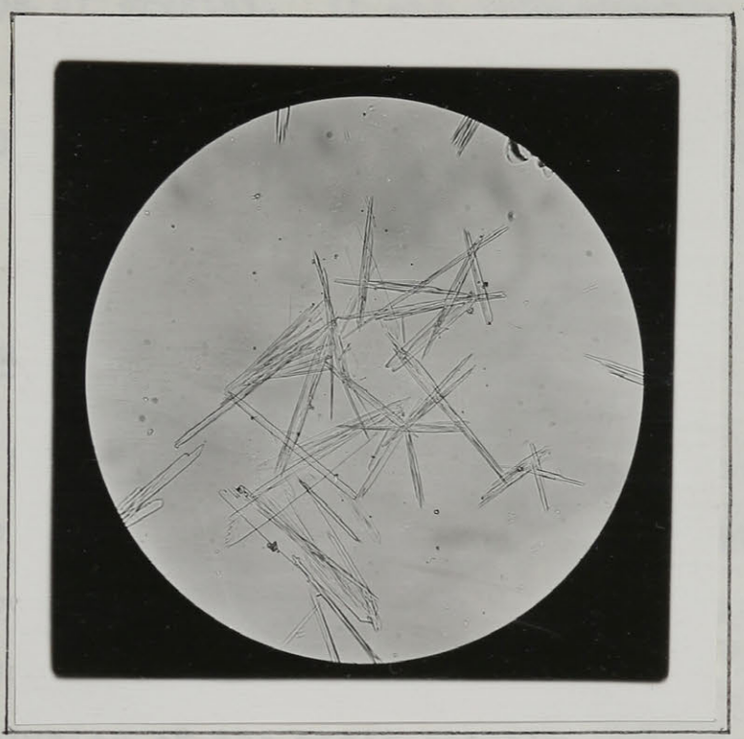
separation may have been only a matter of saturation of the water with one type of substance. The water-insoluble fraction seemed however to be definitely insoluble even when more hot water was added.

The water-insoluble residue was redissolved in ethyl acetate and the process repeated. The crystals again separated on taking off the ethyl acetate. The water was heated and the precipitate centrifuged off. The supernatant fluid from the various crystallizations was concentrated in vacuo and the concentrate heated. A part of the material which separated remained insoluble in hot water and was separated and added to the water-insoluble fraction. The liquid above was then chilled and the crystals separating were regarded as being of the hot water-soluble fraction.

The water-soluble crystals were dissolved in 6 cc. 95 per cent ethyl alcohol. They dissolved readily and completely in the cold.

A photograph of the water-soluble crystals is presented below. Those in the picture were recrystallized 3 times from hot water.

Figure 1.



Crystals from Extract A of Human Placenta.

Crystallographic Form.-

Through the kindness of Dr. Graham and Dr. Osborne of the Department of Mineralogy, the writer was enabled to obtain a crystallographic analysis of these and later crystals.

The crystals were very small and it was impossible to turn them over on the slide. The indices of refraction were not determined. The crystals were described as follows:- Untwinned. Blocked with notched edges. Orthorhombic, positive. Optical axial angle moderate. Indices probably moderate. Birefringence low. Probable birefringence 0.005-0.010.

Compared with Doisy's theelol this was an entirely different type of crystal. It was orthorhombic, not monoclinic and had a low instead of a high birefringence. However, later on (see Extract 10) on crystals prepared in the same way the opinion was revised.

The Bioassay of the Water-soluble Material.-

Since according to Marrian his crystals had a potency of 9000 mouse units per mg., and assuming the rat-mouse ratio to be 4:1, the potency of these crystals if identical with his, might be expected to be about 2500 rat units per mg. On this basis the alcohol solution of the water-soluble crystals was diluted so that one cc. contained half a unit. This was injected in 6 doses over 48 hours in total doses of 3 and 1-1/2 expected units. A negative response was obtained. Three more animals were injected with a total dose of 2.5, 5.0 and 10 expected units. It was decided to change to Doisy's method of 3 doses in 12 hours which was done in this case. All three were negative.

A dose of 45 expected units was given, this time again in 3 doses over 12 hours. A positive response was obtained. This, by calculation gave a potency of only 33 units per mg. This is obviously only a very approximate value but we believed that it showed that the crystals were relatively inert compared to a compound which should have a unitage of 2500 units per mg. The inertness of the material was a disappointment.

It was thought that the potency might all reside in the hot water-insoluble fraction. This was dissolved in alcohol and given in doses up to 12 expected units. All the animals were negative. At this point the work on the material was stopped and it was left in the ice box in alcohol for some months. It was regarded as an inert compound slightly contaminated with oestrin.

At this time Dr. Collip was working with emmenin which was believed to be inactive in the adult castrate animal and to be ether insoluble for the following reasons. Ether extraction of the emmenin fraction acidified with acetic acid took out a large amount of active material in the first few washings. In fact, the ether extract was as effective in terms of weight of original placenta as the crude material had been before extraction. The emmenin fraction was extracted 25 times with ether. In spite of this, after the fifth washing no potency appeared in the ether even when large doses were given. However, after an emmenin fraction which had been extracted many times with ether was treated with ammonium sulphate and absolute alcohol, a large part of the potency was lost to ether extraction. This suggested to the author that emmenin might on occasion pass over into the ether extract. This suggested in turn the assay of the water-soluble crystals on the baby normal rat by mouth. On the basis of a unit being 1/33 mg. the crystals were diluted and total doses of 1-1/2, 3/4 and 3/10 units were fed over a period of three days. In terms of weight of crystals, these doses were 50γ, 25γ and 10γ. The three animals showed a positive

response. A larger series of animals were tested on doses of 10γ, 3γ, 1.0γ, 0.5γ, 0.3γ, 0.15γ and 0.07γ. Full cornification was obtained down to a dose of 1.0γ. The doses of 0.3γ and 0.15γ showed epithelial responses. The animals receiving 0.5γ and 0.07γ were negative as also were those on 0.07γ.

The crystals were then tested on immature ovariectomized animals to see whether removal of the ovary had any influence on the response. The doses fed were 30γ, 10γ, 3γ, 1γ and 0.5γ. Those down to 10γ were positive; below this negative responses were obtained. This appeared to be a result suggestive of ovarian influence on the preparation but the small number of animals made any conclusion impossible. In view of these experiments a further assay on adult castrates was made. It was decided to inject them once a day for three days so as to spread the dose out as Doisy did with his immature rats. Three rats were put on each dose. All animals were negative on doses from 0.5-5γ. Further assays on baby normals showed 11 rats negative on 0.4γ, 2 negative on 0.8γ and 4 positive on 2γ. 4 baby castrates were negative on 2.0γ.

When both water-soluble and water-insoluble fractions were found to be relatively inert it became very difficult to account for the activity which had been present in the ethyl acetate some time before the crystals were obtained. As a bare possibility the two fractions were recombined in case they might then regain the potency they had before separation. No sign of any increase was obtained. In the light of present knowledge, it is difficult to

know whether any real change occurred because of the alteration in the method of assay and the use of such a small number of animals.

From these results it seemed that the unit for the baby normal was of the order of 1γ; for the baby castrate it was between 3 and 10γ and definitely more than the baby normal unit. For the adult castrate using 4 animals on each dose and giving one dose a day for 3 days, it appeared to be about 15γ for a 50 per cent response.

Thus these crystals were 15 times more active on the baby normal than on the adult castrate. They were also 15 times less active than Doisy's theelol on the castrate and also less active than theelol on the baby normal. The units for the two substances cannot be directly compared however,as the methods of assay are entirely dissimilar especially for the baby normal where Doisy's criterion is the opening of the vagina within 10 days of giving the dose,and ours is a positive squamous flush within 3-4 days of the first dose. Dr. Doisy was kind enough to give this laboratory some of his crystalline theelol for comparison with the crystals prepared by us. A comparison of the two will be given later.

In order to facilitate description of the method of preparation and its modifications, it may be divided into steps.-

Step 1. Extraction with ether.
2. Saponification with KOH. Pass in CO_2.
3. Extraction with ether.
4. Extraction with cold acetone.
5. Extraction with boiling 50% alcohol.
6. The cycle ether-aqueous-alkali-acidified extraction with ether.
7. Precipitation with cold ether.
8. Boiling with 'norite'.
9. Recrystallization from dilute alcohol.

The method will not be described in detail again but any modifications will be noted with the step which they affect.

Extract B.

The ether concentrate from 25 kilos of human placenta was used. The saponification and treatment with CO_2 (Step 2) was omitted. Also steps 3, 4 and 5 in an endeavour to shorten the procedure. The ether extract of the emmenin fraction was concentrated to water and acidified with sufficient HCl to make the concentration of the acid 1N. The volume was 600 cc. and the material was active on the immature animal in an equivalent of 1 gram of original placenta; that, there were 25000 units. The acid solution was extracted 10 times with ether, the ether reextracted with 5 per cent KOH, the alkaline solution rendered strongly acid with HCl (about 1N) and then extracted 9 times with ether. The ether was washed and distilled in vacuo. A small quantity of alcohol was added and 5 cc. of redistilled ether. The solution was chilled to

-15°C. in an ice-salt mixture. No precipitate formed. The ether and alcohol were evaporated off and the residue dried in a vacuum dessicator. One cc. of dry ether was then added and the solution was again chilled. A precipitate formed, it was washed with cold ether and boiled with norite in alcoholic solution. It was then dissolved in ethyl acetate, water was added and the ethyl acetate distilled off in vacuo. An insoluble residue formed which did not go into the water on heating. A little alcohol was added and solution was effected. On chilling crystals separated out again. The weight of the crystals (using a micro-balance) was 0.18 mg. This is a much poorer yield in terms of original weight of placenta than the first preparation. It is felt that most of the loss takes place in the cold ether precipitation, a good proportion of the potency remaining in the oil. (See Extract 1 below).

The crystals also differed in being apparently hot water-insoluble. The amount was insufficient for a melting point. The assay on normal immature rats by feeding, using 4 animals on each dose, showed no response below 2γ and on 2γ there were two epithelial responses. The adult castrates were injected once a day for three days. They showed an epithelial response on 5γ, negative on 7.5γ and all positive on 15γ and up. According to this assay these crystals seemed to have very little ratio between the adult castrate and the baby normal.

Extract C.

The rest of the old 300 kilo concentrate was used in this extract. Since Marrian (1930)b had shown that most of the loss in his process occurred in step 2, that is saponification with ether and passage of CO_2 into the mixture with subsequent extraction with ether, it was thought that the ether extraction at this point might not be effective in removing the hormone. It was therefore decided to try acetone as an extraction medium in step 3. This was done as follows. An equal volume of acetone was added to the KOH-CO_2 solution. A uniform solution was obtained. This was allowed to stand. Then the solution was saturated with sodium carbonate. This caused the acetone to separate out. It was hoped that as the hormone had been shown to be insoluble in aqueous sodium carbonate (Marrian, 1930)[a] it would be efficiently carried over into the acetone. The acetone was evaporated and the method was carried through as in Extract A. At step 7, no precipitate was obtained on chilling the ether solution. On testing the oil no activity could be detected. It was concluded that this modification was unsatisfactory.

From this time on, the extracts were numbered and the fractions were given designating letters. In connection with all these preparations an important source of danger may be pointed out both as regards loss of the hormone and danger to the experimenter.

This is the formation of ether peroxides which tend to explode on detonation or on heating. It became the practice to add water to the flask when ether was to be distilled in any quantity so that it was not distilled to dryness. We have had only one explosion which could be definitely attributed to peroxides and one where we suspected this as the cause. In the first case, the boiling point of the ether was being determined. After all the ether had distilled over there was left an oily residue. An explosion took place some seconds after the ether had all distilled over and the source of heat had been removed.

Extract 1.

The emmenin concentrate from 232 kilos of placenta from a commercial source was used and extracted with ether 6 times in 12 litre flasks. The crude ether extract was positive on the equivalent of 5 gm. of original placenta in the baby normal by feeding and was positive on 6 gm. in the adult castrate by injection. It was negative in lower doses on both, thus showing no ratio. The method was the same as in Extract B, that is, the steps in Marrian's process down to the cycle (Step 7) were omitted. It may be noted that much of the acetic acid used to keep the

emmenin acid during ether extraction went into the ether and it required a large amount of 5 per cent KOH to neutralize it before the colour of the ether changed to brown. The cycle KOH acid ether, etc. was repeated twice. The ether after this double cycle showed a positive response on an equivalent of 5 gm. in the baby normal. The ether was evaporated off and the oil transferred to a centrifuge tube, 7 cc. ether added and the tube chilled to -15°C. A precipitate formed which was treated with norite as before and dissolved in ethyl acetate. Some of the precipitate was insoluble in ethyl acetate but was readily soluble in water and left an ash on heating on platinum foil. The ethyl acetate soluble material was assayed and was negative on the highest dose given to the baby normals. However, the preparation was continued. The ethyl acetate was removed in vacuo and the water phase contained a flocculent precipitate yellow in colour. On heating this turned to oil. . The oil was removed and the water set aside in the ice box. No crystals formed.

The residual oil from the cycle was extracted with 50 per cent alcohol and the alcohol soluble part was again treated with cold ether. No precipitate formed. The oil was then dissolved in a large volume of ether and allowed to concentrate slowly at room temperature. Ether was then added to the oil and a precipitate formed. This was treated as usual and a considerable quantity of the water-soluble ash containing material was obtained. It was negative on assay. The ethyl acetate was taken off and a flocculent

white precipitate obtained. This consisted of semi-crystalline very fine needles which were somewhat curved, weight of crystals 0.5 mg. On assay it was negative up to 4.2γ on immature rats and up to 12.6γ on adult castrates. The combined alcohol mother liquors were also negative in all doses given to both castrate and immature animals.

The oily residue was then assayed. It was positive on 8.8 gm. of original placenta in the baby normal by feeding and on 8.8 gm. with an epithelial reaction at 4.4 gm. on the adult castrate by injection, showing that it had lost little potency as compared to the crude ether extract. It still showed no ratio between adult castrate and immature normal animal. This oil was later partitioned between 70 per cent alcohol and benzene and the benzene washed with 60 per cent methyl alcohol. The ethyl alcohol and benzene fractions showed activity on both castrate and immature animal in about the same dose on each. In the benzene fraction, on concentrating crystals appeared but it was found impossible to separate them from the oil. The benzene soluble material contained about 7,000 units and the 70 per cent alcohol 15,000 units; neither showed any ratio between adult castrate and baby normal. The benzene soluble fraction was later used for attempting vacuum distillation and for priming animals.

This equal division of the potency between the benzene and alcohol is interesting in view of the behaviour of Extract 3, where on partitioning the crude ether extract between benzene and 70

per cent alcohol and washing the benzene with 60 per cent methyl and also with 60 per cent ethyl alcohol, all the potency remained in the 70 per cent alcohol and the benzene was negative on the baby normal. This indicated that if Butenandt is correct as to the separation of the keto-hydroxy from the tri-hydroxy in this way, that in the first case the two were present in about equal quantity with a rather larger amount of the tri-hydroxy form and in the last case the tri-hydroxy form was the only one present in the crude ether extract. The absence of the keto-hydroxy form has not been reported for pregnancy urine. It is possible that the non-ether extractable potency in mare's urine is the tri-hydroxy form united to some other compound. It is interesting to note in this connection that Butenandt reports (1931)[a] that Dohrn and others have succeeded in isolating a crystal like his low potency (M.P. 276°C.) compound from the urine of pregnant mares. Extract 3 was not purified further.

Extract 4.

At about this time the conception of the crystals as related to the keto-hydroxy form in a similar way to the tri-hydroxy form but with further changes in the molecule began to be considered. It was shown by Doisy that the theelin fraction was extractable by

ether from aqueous alkali at pH 9-10, whereas Marrian had found it impossible to extract any of his active material by ether from aqueous alkali; also Doisy prepared theelol from the alkaline solution left behind after ether extraction. It was thought possible that the various compounds could be fractionated from each other by extracting with ether, first at pH 8.0,then at pH 5.0, and lastly at approximately pH 2.5. If the water-soluble crystals were in a series with the other two and at the opposite end of the series to the ordinary keto-form,one would expect it not to be ether extractable at say pH 5.0 where most of the ordinary tri-hydroxy form would pass over into the ether. It was hoped to separate the substances in this way.

150 kilos of placenta were treated by the emmenin process and ether extracted in the ordinary way acid to acetic. It may be noted that if the emmenin were related to the crystals, it should on this hypothesis not be ether extractable at the acidity attainable with acetic. However the crude emmenin is not ether extractable even after boiling with strong HCl. It was hoped that the large volumes of ether used would extract the water-soluble crystal at the pH since it was impossible to acidify the emmenin with HCl.

The ether extract was evaporated and water added. 25 per cent was added to make the solution just alkaline to phenolphthlein (pH 8-9). It was extracted 5 times with ether. HCl was added to the solution to make it about pH 5. It was extracted 8 times with ether and more colour came across into the ether. A greenish

fluorescence was noted in the ether extract. The residual solution was then acidified to pH 2.5 with HCl and extracted 5 times with ether. The water residue after all the ether extraction was neutralized to pH 7 and concentrated to a syrup, alcohol was added to precipitate the salt formed in the reaction of the KOH and the HCl. Water was added and the ether was evaporated from the extracts. The water was made slightly alkaline and the precipitate formed when the ether was removed, redissolved.

The first ether extract made at pH 8 was assayed on adult castrates. The assays at this time were being made by a new division of the dose into two doses a day for two days, that is, four doses over a period of 36 hours. The ether extract at pH 8 was active in the equivalent of 2.5 gm. of original placenta on the adult castrate. There was no ratio, the baby normal was positive on 2 gm. and negative on 0.8 gm.

The ether extract at pH 2.5 was negative in baby normals up to 770 gm. by feeding. The water residue was also negative in all doses on baby normals.

A summary of the experiments carried out with the extract at pH 5.0 is given in the table. (Table X)

TABLE X.

Assay of Ether Extracted at pH 5.0.

Test Animal	No. of gm. of original placenta		
	Squamous Reaction	Epithelial Reaction	Negative
Immature Normal			
#Injection	770	320	less than 320
Feeding	46. 30	-	32
Immature Castrate.			
#Injection	770	264	less than 254
Feeding	330	150	less than 150
Adult Castrate.			
#Injection	1540	770	less than 770
Feeding	*1540		less than 1540

4 injections in 36 hours.
* 1 animal positive. Repetition on 6 animals all negative.

As will be seen the extract at pH 5.0 was positive by feeding on the baby normal in a comparatively small dose. It was negative on both the immature and castrate rat by injection up to large doses 25-50 times that for the oral route on the immature rat. It showed equally low potency on the adult castrate by mouth and by injection. The immature castrate rat showed an oral unit intermediate between the adult castrate and the immature normal.

It was thought at this time that the inactivity by injection on all types of animals indicated the presence of an inhibitory substance which was destroyed or not absorbed by mouth. The inactivity on the adult castrate by mouth was explained as meaning that the active substance as well as the inhibitor was not readily

absorbed. It is difficult even now to explain the inactivity on the immature rat by injection. In later work it was possible to obtain almost equal values for the amount of material required by mouth and by injection. This was however with crystalline material. In connection with the idea of inhibition the pH 5.0 ether extract was injected together with various oestrogenic substances in order to detect any inhibition of their action. A.P.L., emmenin and theelin were tested in this way. No evidence of inhibition was found. Since however there was some activity in the pH 5.0 ether extract itself, it was thought that the inhibitory and active substances in the extract might be almost evenly balanced. It was therefore decided to use the pH 2.5 ether extract which had been proved to be negative on large doses by mouth in the immature normal animal. It was injected in large doses together with A.P.L., emmenin, and theelin. No inhibition was observed in the first two but the action of the theelin did not appear. The number of units of theelin given with the ether extract was later increased. The first positive were found at a dose of 90 units of theelin. This seemed to indicate an inhibition of the theelin action. However, it was found that muscle extract prevented theelin acting up to 20 units by injection. Also when the theelin was given by mouth and the pH 2.5 extract injected, the theelin gave positive reactions in doses of 6 units and up. The units of theelin given here are adult castrate units as assayed by the laboratories at St. Louis for Parke, Davis and Company. The theelin used was this company's

preparation. As Doisy has shown, the dose for the immature is 3 adult castrate units, so that a positive reaction on 6 units shows no inhibition. The pH 2.5 ether extract was also digested with trypsin in an endeavour to destroy the hypothetical inhibitory substance. After digestion the dose by mouth on the immature rat had not changed and the extract showed no more activity by injection on either type of animal than before. The hypothesis of an inhibitor had therefore to be discarded. Before this conclusion was reached, an attempt was made to induce pseudo-pregnant reactions in the castrate rat uterus on the theory that the inhibitor might be related to the corpus luteum hormone. The extract at pH 2.5 was injected twice daily for 5 days into adult castrate rats. Theelin was also injected into some of these animals as it has been shown to sensitize the uterus to the action of the corpus luteum hormone. After the period of injection, a black silk thread was passed through the wall of the uterus and the injection continued for one more day. At the end of 4-6 days the uterus was examined. In no case was there any sign of a deciduoma. There were a few thickenings around the thread but on microscopic examination they proved to be merely an inflammatory reaction. There was therefore no substance capable of producing the pseudo-pregnant reaction in the castrate present in the pH 2.5 ether extract.

In retrospect the assays on this extract while satisfactory as far as the division of the dose is concerned, suffer from the small numbers of animals used for each dose. In the immature normal

a number of indefinite epithelial reactions were observed at doses considerably below those at which a positive reaction appeared.

The irregular results obtained on feeding and injecting theelin in the investigation on Extract 4 led to an attempt at assaying the commercial theelin preparation.

In order that the assay should be strictly comparable to Doisy's we changed the number of doses to 3 in 12 hours for the adult castrates and to one injection daily for 3 days for the immature animal. The feeding was carried out in the usual way over 3 days.

The results are presented below. (Table XI)

TABLE XI.

Bioassay of Theelin. (Parke, Davis & Co.)

Test Animal	Dose (Units)		No. of Animals	
			Positive	Negative
Immature normal.				
Injection	3,9,15,21,30,45, 60	(each)	- 1	1
Feeding	1		-	5
	2		1	4
	3		9	3
	5 and 10		3	-
Immature castrate.				
Injection	4,10,15,20,40,60	(each)	-	1
Feeding	3		3	-
Adult castrate.				
Injection	2		-	30
	4		-	6
	8		-	6
	20		-	11
	40		-	2
	60		-	3
Feeding	5,10,15,20,25,	(each)	-	1

THe results with the commercial theelin were very disappointing. The only uniform response obtained was that of the immature normal by feeding. This value of 3 castrated units for an immature rat unit checks exactly with Doisy's claim for theelin, but as mentioned above, the results are not really comparable, due to difference in criterion of response. This showed that the theelin had not become inactivated as far as the reaction of the baby normal

by mouth was concerned. The results by injection with the baby normal were negative up to 45 units, the first positive was at 60 units. It is unlikely that individual variation in the rat would vary 2000 per cent, which is what would be required to explain this result. However, the number of animals is again small. The immature castrate showed the same unit by mouth (3 adult castrate units) as the immature normal. One positive was obtained by injection at 45 units but a negative was obtained on 60 units. The adult castrate rat by mouth showed no response up to 25 units. Doisy has not tested this, but the result is not surprising in view of the great variations in value claimed for oestrin. The adult castrates by injection are most difficult to explain. Here a large number of rats were first primed with Extract 1, and all showed a positive reaction. One week later they received 2 units of theelin in the division of dose used by Doisy. They were all negative. 11 primed rats were put on 20 units and were all negative. There was no sign even of an epithelial response. Smaller numbers of adult castrates were negative up to 60 units. Individual variation in rats cannot account for this result. As shown later when the dose is divided into 6 doses in 36 hours, the commercial theelin has much more potency. This has no bearing however on the fact that using Doisy's method of assay, we were unable to duplicate his results.

It is possible that the particular batch of theelin used decreased in potency since the first assay at the St. Louis

laboratory. The satisfactory potency on the baby normal cannot be explained on a loss of potency unless one regards the theelin as being converted into a form active on great division of the dose, where perhaps the body is given an opportunity to convert at least part of the inert product back to the active form, whereas in the case of injection over 12 hours or in single doses for 3 days, the substance might be so quickly excreted or destroyed that no opportunity would occur for conversion. This would also agree with the greater potency obtained on dividing the dose still further in the adult castrate and giving it over a longer period of time. The castrate and normal immature rat appear to absorb and utilize the theelin with equal efficiency since the doses for a positive response are the same. When the theelin was fed to the normal immature rat by pipette once a day for 3 days, using 2 rats for each dose negative results were obtained with 4, 6 and 10 units. This indicates that the oral route is not by itself the cause of the better response by the immature animal, but that it is the great division of the dose resulting in almost continuous slow absorption. On the theory that the intestine or liver of the immature rat might act to convert the hypothetical inactivated theelin back to the active form, the theelin was incubated at 37°C. for one hour with saline extract of liver and intestine from immature rats. This was then injected into immature rats as before. The theelin was inactive on a dose of 7 units so that the treatment had not increased its potency by this method of injection to equal that which it had by

mouth. The liver and intestinal extracts gave a negative response when injected.

The fact that the pH 5.0 extract did show a ratio between the dose for adult castrate and the baby normal animal, encouraged the attempt to prepare a purified material from it which should have a ratio similar to that of the first crystals. Several extracts were made in this way, first extracting with ether at pH 8 and then acidifying to pH 5.0 and re-extracting with ether. They were also tried for their hypothetical inhibitory content. However, no satisfactory results were obtained. A further assay of theelin will be presented later.

Extract 9.

In Extract 9, the ether concentrate from the emmenin process on pregnancy urine was used. The urine was concentrated and precipitated with alcohol, the alcohol concentrated to a paste and extracted with acetone. The acetone was evaporated and water added. The water solution was adjusted to pH 8.0 and extracted with ether. Since the ether extraction had been made at pH 8.0, most of the active material in it might be expected to have no ratio (See Extract 4), though this extract is not strictly comparable to Extract 9 since the latter was derived from pregnancy urine and the former, placenta. The ether extract was concentrated to water and extracted

3 times with benzene, the benzene was distilled off and the residue steam-distilled as in Doisy's preparation of theelin. It was leached with 0.25N NaOH. The alkaline solution was extracted with ether without previous neutralization. Very little colour passed into the ether. On evaporation of the solvent a light brown oil was obtained which contained numerous crystals. These dissolved in alcohol with difficulty, those not readily soluble in this solvent were removed. Water was added to the alcohol and a white precipitate formed. This was left over night in the ice box. The crystals were centrifuged off and redissolved and reprecipitated. They were negative on assay by feeding to immature rats. The mother liquors were also negative. On tracing back, the potency was found to have been left in the alkaline solution, so that with this type of preparation it is not possible to extract any of the active material from 0.25N alkali into ether. Doisy says later that the pH of the alkaline solution during extraction is between 9 and 10. The strength of alkali which he uses is 0.25N and this gave a pH of 11-12 in the case of the above extract. He makes no mention of neutralization but it is difficult to see how he obtains the required pH without doing so unless his residue is strongly acid, and there is nothing in his method to produce this condition.

Attempted Distillation in High Vacuum.-

After the series of unsuccessful attempts to duplicate the first crystalline preparation, it was decided to try the method of

high vacuum distillation. The Langmuir pump was made by Dr. Wright, at that time in the Department of Physical Chemistry. The author is greatly indebted to him for this kindness. The distillation was carried out according to Butenandt's directions. A CO_2-acetone trap was employed. The apparatus was all glass sealed. The material to be distilled was placed in a small glass tube with an expanded bulb. The tube was sealed on to the apparatus. The McLeod gauge was not calibrated but the order of vacuum obtained was below 0.001 mm. and probably below 0.0001 mm. The Langmuir pump was backed by a Cenco Megavac pump. The oil from the benzene soluble fraction of Extract 1 was first distilled. The retort containing the material was surrounded by an air bath, electrically heated, and the outside temperature was 100-115°C. for the first 24 hours. A light brown oil distilled over slowly. The vacuum was broken and this was washed out with cold ether. The apparatus was resealed and the temperature was gradually raised to 150°C. in the next 36 hours. A light yellow oil distilled over but no crystals appeared. This fraction was removed and the temperature raised again slowly to 210°C. More light yellow oil distilled over. The residue was very dark and quite dry. No crystals appeared in the distillate. The 3 fractions were tested on the immature normal rat by oral administration. All three were active in about the same dose. There seemed to have been no concentration of the hormone in any one fraction. According to Butenandt, most of it ought to come over at 130-160°C. An extract from pregnancy urine made by Dr. Collip was next distilled. Only two fractions

were tested for potence. The one coming over from 100-115°C. and the other from 130-140°C. were both negative. The distillate consisted of a pale yellow oil with a small quantity of a solid white material which was not definitely crystalline. It is felt that the reason for the failure to obtain crystals by distillation was due partly to the small quantities which were used. Only a very few milligrams of active crystals need have been present in the oils to account for their potency before distillation.

Extract 10.

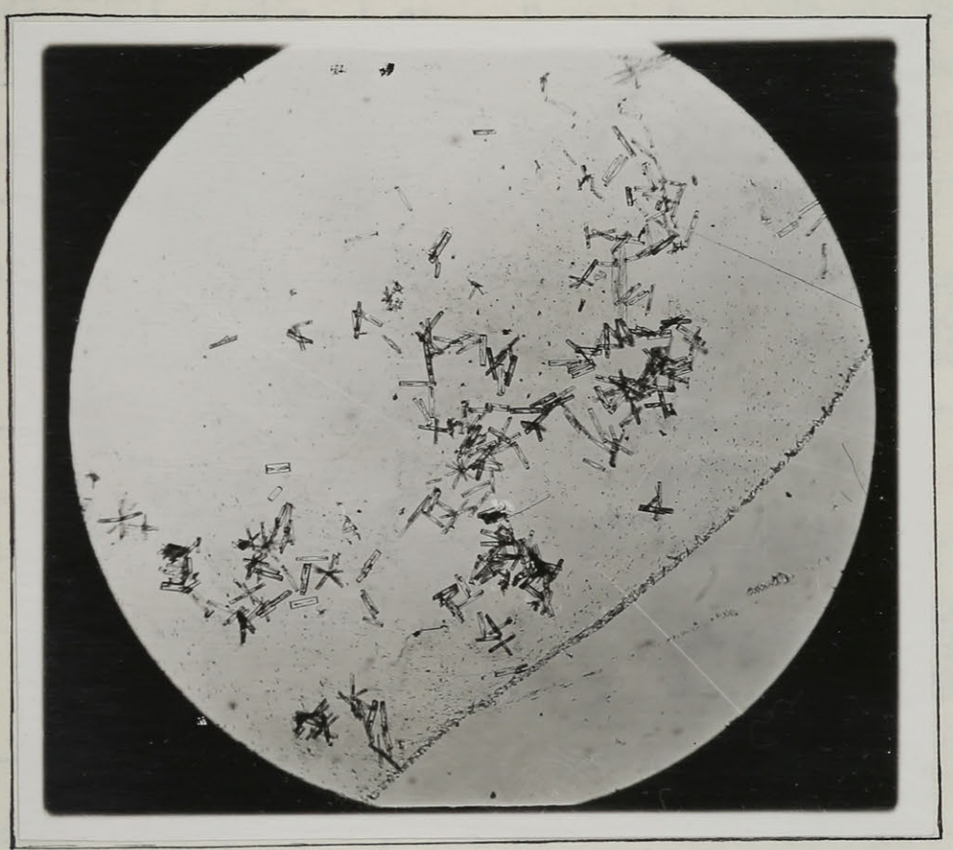
It was next decided to duplicate as nearly as possible the conditions of the first preparation. The emmenin concentrate from 445 kilos of material was obtained, acidified with acetic acid and ether extracted. The ether was evaporated and the residue dissolved in alcohol. The equivalent of 150 kilos was taken and treated immediately, the rest was stored in alcohol at room temperature. The material so prepared was active in the equivalent of 4 gm. original placenta, that is about 35,000 units in the 150 kilos taken. The ether extract after the 5 per cent KOH-CO_2 extraction (Step 3) was still active in 4 gm. of original placenta. The procedure was carried out exactly as before. No precipitate separated out from cold acetone in this preparation. The 50 per cent

alcohol insoluble part of Step 5 was negative up to 81 gm. original placenta. All these assays were made by oral administration to the immature rat. The absence of potency in the 50 per cent alcohol insoluble fraction indicates that no keto-hydroxy form is present if Butenandt is correct in his statement that it is at this point in Marrian's process that the keto-hydroxy form is retained in the residue. At Step 7, on adding ether no precipitate was obtained. It was thought that the pH might have something to do with the failure to obtain crystals from the oil. A small quantity of very dilute alkali was added to the oil. The ether was evaporated off and on chilling by means of a mixture of CO_2-snow and acetone a precipitate was obtained. This was insoluble in ether and was crystalline and almost white after washing off the traces of oil. It was later found that evaporation of the ether to dryness and the addition of more ether often induced crystallization after several repetitions. The precipitate was readily soluble in absolute alcohol. The material was treated with charcoal in 95 per cent alcohol, and then dissolved in ethyl acetate as in Extract A. The ethyl acetate was taken off after water had been added. A flocculent precipitate appeared. The water was heated and the precipitate coagulated and settled out. This hot water-insoluble fraction was separated and the water was chilled. A small amount of crystals separated out from it. The hot water-insoluble crystals were dissolved out in alcohol and recrystallized 3 times from dilute 25 per cent alcohol.

The water-soluble and the water-insoluble crystals had the same appearance in this case. A photograph of the crystals is shown below. They can be seen to be quite different from those of the first water-soluble crystals in Extract A. The crystals from Extract 10 were parallelograms. The edges were at right angles to each other. There were some air bubbles included in the crystal as it dried on the slide. These were not present in the material before drying. The total weight of crystals obtained was 4.5 mg. The amount was used for assay rather than for a melting point. The total recovered potency as water-soluble and water-insoluble fractions was 8000 units (immature normal oral administration) or about 25 per cent of the potency of the original ether extract. The early assay of both the water-soluble and water-insoluble fractions were active by feeding to the immature rat in very small amounts and inactive on the adult castrate by injection (3 times in 12 hours) up to about 50 times the dose. The amount of potency in the water-soluble fraction was very small and the material showed the same ratio as the water-insoluble form. It was concluded that they were identical. Later assays were done on larger numbers of animals and with the divided method of dosage.

The assay results on the various types of animal were as follows:-

Figure 2.



Crystals from Extract 10 of Human Placenta.

TABLE XII.

Assay of Crystals from Extract 10.

Test Animal	Dose γ	No. of Animals				Percent Response	
		Total	Full Sq.	Epith.	Neg.	Squam. Sq.	and Epith.
Adult Castrate. Injection							
(3 X in 12 hrs.)	18-72		0	0	4	0	0
(4 X in 36 hrs.)	144		1		1	-	-
(6 X in 36 hrs.)	13.5	21	7	6	8	33	62
Theelol: (for comparison)	2.5	15	8	2	5	53	67
Immature normal. Injection							
(6 X in 36 hrs.)	1.8	6	3	1	2	50	67
	2.50	7	4	1	3	60	60
	3.2	5	3	2	0	60	100
Theelol: (for comparison)	1.25		3		3	50	50
Immature Normal Feeding	1.26	9	5	2	2	55	75
	1.40	6	3	1	2	50	67
	1.80	5	3	0	2	60	60
Immature Castrate Injection: (3 X in 12 hrs.)							
1,3,4,0,27,41,54 γ each.....			0	0	1		
Feeding:							
1,3,2,4,3,6 γ each.....					1		
	7.2		1				
	14.8		1				

In the case of the adult castrates injected with Extract 10 there is a difference between the effect of 3 doses in 12 hours and 6 doses in 36 hours. Though the number of rats in the former group is small, the dose is ineffective up to 5 times the more divided dose. That individual variation can play a part even up to 10 times the dose is seen in the one rat negative on 144γ when the dose was divided into 4 doses in 36 hours.

The comparison on the same group of rats of Extract 10 and crystalline theelol shows that the former is much less effective in producing a positive response in this type of animal. Theelol is about 10 times more effective. The rats in these groups were all primed, or had been positive a week before this experiment was performed.

The immature normal rats by the same method of injection gave a 50 per cent response on 1.8γ and a 60 per cent response on 2.50γ. The number of rats in this series is too small but it suffices to show that the placental crystals are at least 10 times as effective on the immature normal rat as they are on the adult castrate. Theelol administered to immature rats gave a response of the same order as did the placental crystals. By feeding, the immature normal unit was slightly lower than by injection in both theelol and the placental crystals.

These results indicate that crystals from placenta have physiological properties which differ from those of theelol. They are much less effective by injection on the adult castrate and act

in about the same dose on the immature normal rat.

Extract 11.

The alcohol concentrate which was the starting point for Extract 10 was allowed to stand at room temperature for six weeks. At the end of this time it was assayed on the immature rat by feeding. It was negative up to the equivalent of 45 gm. of original placenta. The concentrate had lost nine tenths of its potency on standing. The reason for this is not known. It is possible that there was present in the alcohol some fluorescent substance which sensitized the active material to light destruction. The concentrate had been placed in a north window. The concentrate was allowed to stand in alcohol in an attempt to duplicate the conditions under which the first crystals had been obtained. The ether concentrate used in Extract A, stood in alcohol at room temperature for some months prior to use. It was thought that some change might take place in alcohol solution which would make the crystals more readily isolated.

In spite of this low original potency the purification was carried out as before. Only a very small quantity of crystals was obtained (1.25 mg.). The crystals were very like the first ones (Extract A) in appearance with pointed ends and arranged in sheafs.

They were quite unlike those from Extract 10. The crystals were used for assay. The results were inconclusive as insufficient material was available for the use of the necessary number of animals. Nine adult castrate rats were all positive on 12.25γ and 5 were positive and the other 4 used showed epithelial responses on 24.4γ. The immature rat on 6 animals showed a response of 3 positive on 2.94γ and single animals were positive on 1.71, 1.28 and 0.9γ. The ratio of this substance is much less than that of the original crystals according to this rough assay. It seemed possible that with the great destruction of potency which had occurred the high ratio material had been destroyed to a greater extent than the low ratio substance which must also be present in placenta since a lack of ratio is often found in crude extracts of placenta.

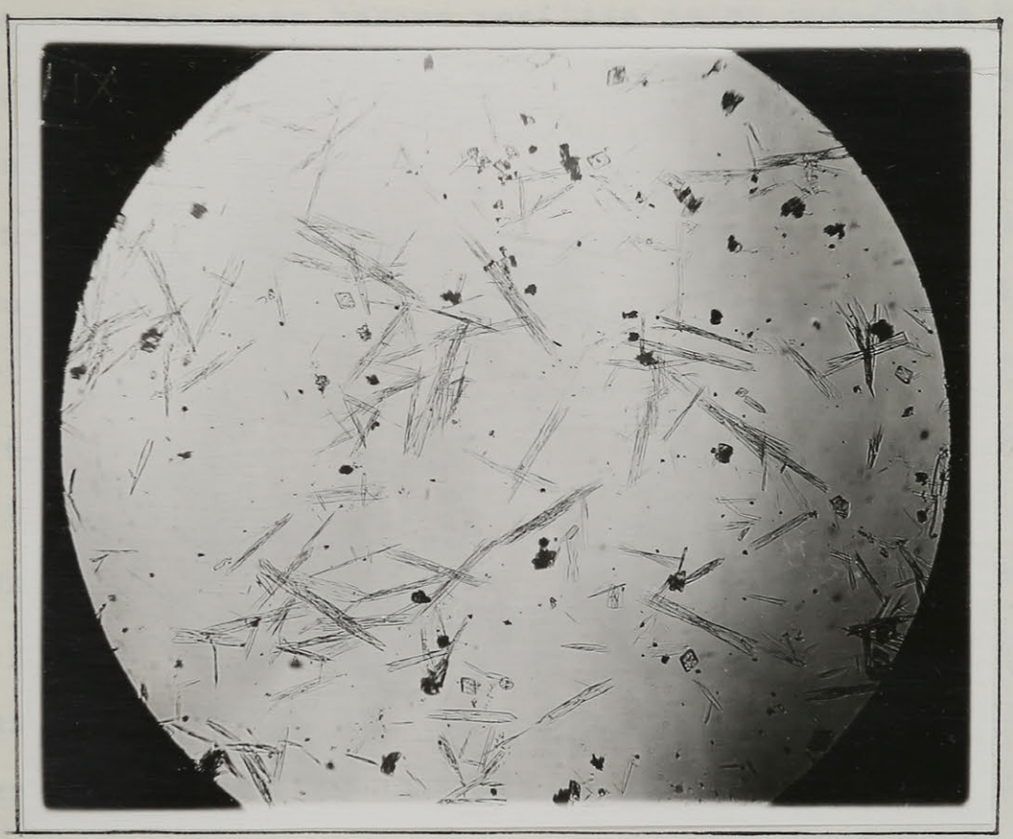
Extract 12.

This was an attempt to carry out Doisy's procedure for the preparation of theelin from pregnancy urine. 60 litres of urine were extracted with ether. His instructions were followed except that when the 0.25N NaOH solution was extracted with ether it was first adjusted to pH 9-10. This, as mentioned above, is the pH which Doisy indicates as the correct one for this procedure, but in his method he gives no indication that he makes the necessary addition

of acid to the sodium hydroxide solution to bring it to this pH. Crystals separated at the expected point and were readily recrystallized from butyl alcohol and petroleum ether and from aqueous alcohol. The yield was 3.2 mg. The appearance of the crystals was not uniform. Some rhomboids and some sheafs of needles similar in appearance to the crystals from Extract A and Extract 11 were seen. The needles predominated. That these rhomboids are not necessarily contaminating material is indicated by the recent preparation by Dr. Collip of crystals of the same type by recrystallization from water of a material in which the other fractions showed an appearance typical of the active material. It is probable that the rhomboid crystals separated out as the material dried on the microscopic slide. The melting point was not done.

On assay on adult castrates in a dose of 7.7γ in 15 animals, 7 or 46 per cent, showed a full squamous reaction and 13 or 87 per cent, showed either squamous or epithelial response. On feeding to the immature normal animal, one out of 7 showed a positive response on 1.28γ and 2 rats out of 4 showed a positive response on 3.84γ. By injection there were 3 out of 6 which showed epithelial reactions on 3.84γ. This material appears to be more active on the adult castrate than the placental crystals and less active on the immature animal. The number of animals is too small for a definite statement. There is very little ratio. However the potency is very low, at least 20 times less than the value given for theelin. When the crystals were assayed in 3 injections in 12 hours, as Doisy does

Figure 3.



Crystals from Extract 12 of Pregnancy Urine.

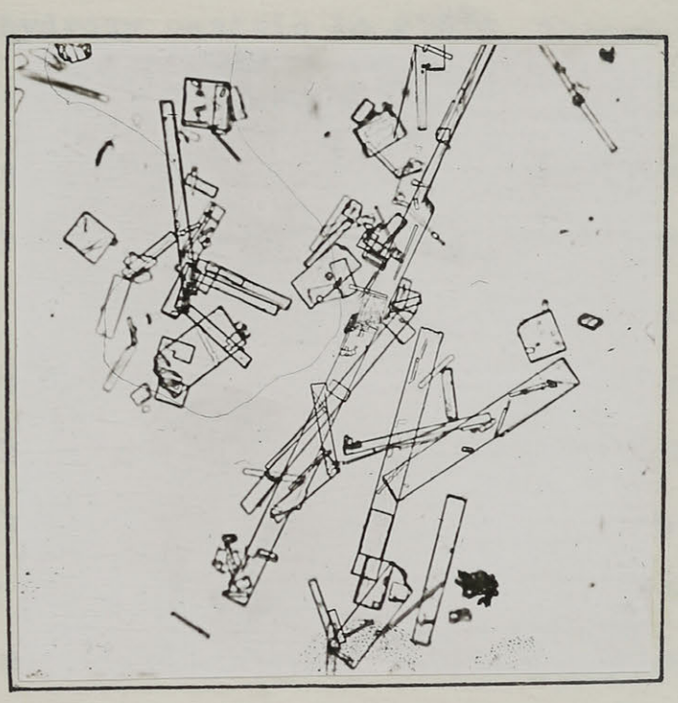
for theelin negative responses were obtained on all doses from 0.96γ to 76.8γ (there were 7 intermediate doses) on rats primed the previous week. One positive response was obtained on 102.4γ.

When one considers that this is 300 times the dose for pure theelin as obtained by Doisy, the degree of individual variation in rats has to be greatly extended if it is to explain the facts. These rats become positive using the crude oil(benzene-soluble) from extract on doses given 3 times in 12 hours equal to those which act in the immature normal rat. It is difficult to think that they can be so extraordinarily insensitive as all the results so far on the response to 3 injections in 12 hours of purified material would indicate. The crude extract was injected in great dilution in watery solution so that the medium is the same as the purified preparation. The inactivity by the three dose method has been shown above to extend to the commercial preparation of pure theelin which is assayed by Doisy's laboratory. It is of course possible that the crystalline preparations are more readily excreted or destroyed than a crude watery preparation. Allan et al (1928) found that 4 injections in 12 hours of a watery crude material was quite inactive on the same dose that showed a 55 per cent response when given in 6 injections in 48 hours, and that it took 40 times the dose to raise the response from 0 to 40 per cent when a single dose was given. Butenandt found his keto-hydroxy oestrin to be 5 times more active when injected in 6 doses over 48 hours than when injected once only. He used an oil medium.

Extract 15.

155 kilos of ether extract of placenta which had stood in ether for some months was next obtained. It was put through the usual fractionation. Considerable difference was noted in its behaviour. In cold acetone a very large amount of crystalline material separated out. This was readily recrystallized from alcohol and gave a positive Liebermann-Burchard reaction for cholesterol. When the ether extract after the cycle was concentrated in a small flask a quantity of white material separated out on the sides of the flask. The material dissolved readily in alcohol and was thought to be cholesterol which had carried over. However, the Liebermann-Burchard reaction was negative. The material was not put through ethyl acetate but was recrystallized 3 times from 50 per cent aqueous alcohol. The material was then nearly white and under the microscope showed a crystalline form very like that of Extract 10. The appearance is shown in the photograph. A preliminary melting point determination showed charring and shrinking at 265°C. and the substance melted at 268°C. with decomposition. The material was recrystallized 3 more times and was then snow white in appearance. Its form is shown in the photograph. The melting point was now 274°C. with slight preliminary darkening at 271°C..The melting point was fairly sharp. At this point a mixed melting point with Doisy's theelol was done. In the melting point apparatus used theelol showed

Figure 4.



Crystals from Extract 15 of Human Placenta.

a fairly sharp melting point at 276°C. The mixture melted sharply at 275°C. For ordinary chemical identity this would be good evidence. However, Butenandt's experience with his inactive form of tri-hydroxy oestrin which gave no change in melting point when mixed with Marrian's active tri-hydroxy oestrin, should be borne in mind. Also, Marrian has recently raised the melting point of his active tri-hydroxy oestrin to 278°C. though he uses a short thermometer, so this is not strictly comparable. Also the melting point of Marrian's A and B keto-hydroxy oestrin is the same.

These points were not known to the author at the time this extract was made. It was felt that probably the compound was identical with theelol and would show the same potency on assay.

The yield of this recrystallized material was 26 mg. It would probably have been about 35 mg. as one third was lost while evaporating the final ether extract. This represents a yield of one fifth of a milligram per kilo of placenta and of 155 baby normal units per kilo. It should be remembered that this only represents a fraction of the potency of the original placenta since most of the activity is ether insoluble in the ordinary way. The yield from this extract is so much better than any previously obtained that any difference in preparation was looked for. The large amount of cholesterol, and possibly other crystalline substances, is the point of similarity between this and the first extract. All extracts between these showed no precipitate at all with cold acetone, that from the first extract also showed a positive Liebermann-

Burchard reaction. This ether concentrate was made by a commercial firm. The presence of the large amount of cholesterol suggests that they used a higher percentage of acetone in the original extraction of the placenta. The original ether extract was positive in 2.00 gm. when tested on the immature normal animal. This gives the total number of units as 77,500; about 26,000 units were recovered as crystalline material and about 35,000 would have been recovered if the accidental loss had not occurred at the last step. This is a 50 per cent recovery. It should be noted that any potency in the original ether extract due to keto-hydroxy oestrin will be lost.

Through the kindness of the Department of Mineralogy, a crystallographic analysis of these crystals was done. The crystals were rather larger than those of the first extract. However, they tended to lie all on one face and to show characteristics of the orthorhombic system on this face. It was found possible to turn them over however and they showed the following characteristics.- Very thin plates lying mostly so as to give impression of being orthorhombic. System, monoclinic. Habit, bladed. Optically positive. Birefringence strong on the face which was usually visible, low on the other face. Refractive indices, α 1.54, β -, γ 1.68. The crystals show inclined extinction of 7°. Only one type of crystal is seen. It is possible that the crystals first examined (Extract A) were identical with the present ones and that the low birefringence found there was due to all the crystals lying

on the other face just as they all lay on the face with high birefringence in this case. It is understood that such differences in crystal habit are not uncommon. Comparison with Doisy's theelol shows the latter to have an inclined extinction of 5°. It differs in being optically negative and therefore shows an interference figure. This is not shown by the crystals from Extract 15. The theelol has a high birefringence and is monoclinic. The refractive indices are quite comparable with those for Extract 10.

In contrast to this evidence for identity stands the difference of physiological potency.

Before discussing the assay of Extract 15, it may be mentioned that another extract has since been prepared from placenta. Seven milligrams of pure white crystalline material was obtained. The crystal form was the same as that of Extract 15. The crystals showed preliminary darkening at 270°C. and melted at 274°C. A carbon-hydrogen determination on this material has been done by the Research Service Laboratories at New York. A preparation (Extract 42) from pregnancy urine melting at 275°C. prepared by Dr. Collip was done at the same time.

	Carbon Percentage	Hydrogen Percentage
Extract 17:	74.8	8.8
Extract 42:	74.8	8.9

The agreement between the analysis of the two crystals from different sources is interesting. If one compares the analysis

with those done by others on the tri-hydroxy form of oestrin to which this compound is closely related it will be seen that the carbon content checks quite well but that the hydrogen is higher in these analyses than in any of the others. Marrian gives 8.27 for his latest preparation, Butenandt gives 8.33, 8.23, 8.39, for three analyses. Doisy gives 8.38. An analysis of Doisy's theelol by the same laboratory (Research Service) gives 8.20 per cent. The hydrogen determination is liable to considerable errors. This value would correspond roughly to the introduction of two more hydrogen atoms into the molecule and so perhaps to the saturation of one of the double bonds. Very much against this interpretation is the fact that the melting point of the compound is much the same as that of theelol and that a mixed melting point gives no lowering. Further analyses both of the substance and its derivatives would have to be done before any definite statement could be made.

Assay of Crystals from Extract 15 and a Comparison of their Physiological Properties with those of Theelin and Theelol.

These physiological investigations have been carried out on Extract 15, on the crystalline theelol kindly supplied by Dr. Doisy and on the commercial preparation of theelin made by Parke, Davis and Company.

A table of the results of assay of Extract 15 on different types of test animals and by different methods of administration is given below.

TABLE XIII.

Comparison of Crystals of Extract 15 with Crystalline Theelol.
(1) On Adult and Immature Ovariectomized Animals by Injection.

Test Animal	Age	Dose γ	No. of Animals Total	Full Sq.	Epith.	Neg.	Percent Response Sq.	Sq.and Epith.
Adult Castrate. Injection		11.0	28	6	7	21	21	25
		14.6#	13	0	4	9	0	31
		20.5	28	15	6	7	54	71
		20.5	12	9	1	2	75	83
Theelol: (for comparison)		2.5	15	8	2	5	53	67
		3.0	14	6	1	7	43	50
		5.0	21	17	3	1	81	95
Adult Castrate. Injection		15.7	100	62	20	18	62	82
		1.57	100	80	8	12	80	88
Immature Castrates Injection		4.4	11	1	0	10	9	9
		6.2	11	2	2	7	18	36
(Castrated 21 dys)	23	9.5	10	2	3	5	20	50
	25	10.0	11	4	1	6	36	45
	50	10.2	15	11	2	2	73	87
	23	12.0	10	7	1	2	70	80
Theelol: (for comparison)	26	2.43	20	0	1	19	0	5
	32	3.24	20	11	2	7	55	65
	21	1.62	6	1	0	5	16	16
	32	2.43	12	6	2	4	50	66
Immature Castrate Injection (9 X in 48 hrs.)	32	6.2	8	7		1	88	88
	25	8.4	12	10		2	83	83
Feeding	25	2.6	8	5		3	63	63

These rats had not been used for a month before this experiment.

TABLE XIV.

Comparison of Crystals of Extract 15 with Crystalline Theelol.
(2) On Immature Normal Rats by Injection and Feeding.

Test Animal	Dose	No. of Animals				Percent Response	
		Total	Full Sq.	Epith.	Neg.	Sq.	Sq. and Epith.
Immature Normal Injection (21 days old) (6 X in 36 hours)	1.13	10	0	0	10	0	0
	1.21	13	7	3	4	54	70
	1.32	12	3	1	8	25	33
	1.55	30	14	4	12	47	60
	1.65	10	0	1	9	0	10
(9 X in 48 hours)	1.15	10	4	2	4	40	60
Theelol: 0.1, 0.15, 0.2, 0.3, 0.7 (each)		1			1	-	-
	1.25	6	3		3	50	50
	1.62	9	4	5	0	45	100
	2.50	6	4		2	68	68
Immature Normal Feeding	0.92	10	3	3	4	30	60
	1.00#	20	2	3	13	10	35
	1.80	11	9		2	89	89
	2.00#	20	3	2	13	15	35
Theelol:	1.32	10	3		7	30	30
	1.50	5	5		0	100	100

These 2 assays were on the same group of animals.

The comparison of the effects of theelol and Extract 15 on the adult castrate animal by injection shows a definite difference in physiological potency between the two. The amount of Extract 15 needed

to produce a 60 per cent response on recently castrated adult animals is 15.7γ whereas the amount of theelol necessary for an 80 per cent response on the same animals is only one tenth as much or 1.57γ. On older castrates the amount of each substance necessary to obtain a response is definitely raised but there is no significant change in ratio. The weight of theelol required for a response is higher than that reported by Doisy, which is 0.68γ for a 75 per cent response. It is of the same order, however, whereas the potency of Extract 15 is at least 25 times less than the reported value for theelol. One cannot compare the values, for, as mentioned before, Doisy gives his injections 3 times in 12 hours. When his method of injection was used, but only on a small number of animals all were negative up to 12γ. The substance was not tested further in this manner. The large number of animals used in the comparison and the strictly comparable method of assay of the two products gives confidence in the reality of their difference physiologically on the adult castrate.

On the immature normal rat the two substances have approximately the same potency. It will beseen that the results on the immature animal even using fairly large numbers of animals vary considerably. The possible reason for this will be discussed later.

On the immature castrate the dose of Extract 15 required is several times greater for a satisfactory response than in the immature normal animal. Thus the difference in response between

the adult castrate and immature normal rat is not due to difference in weight. The immature castrates were used within 2 or 3 days of castration so that no degeneration of the accessory sex organs had time to occur. The results with the immature castrates are much more regular than those with the normal animals. That the operative procedure and cutting the uterus is not responsible for the decrease in sensitivity after castration in the immature animal, is shown by the response of a control group of 6 rats which were operated upon at the age of 21 days. The uteri were cut and pulled on but the ovaries were not removed. These rats showed a 100 per cent positive response on 1.5γ of the crystals from Extract 15. It is felt that the difference in response between the immature normal and the immature castrate indicates that the ovary even when immature plays a definite role in the difference of effect between theelol and the crystals prepared from human placenta. The effect of castration on the action of theelin (P.D.) will be discussed later.

This difference of effect between the immature castrate and the immature normal animal tends to disappear as the division of the dose is increased. Thus, when 9 injections in 48 hours are given, a dose of 6.2γ is sufficient to produce an 88 per cent response in the immature castrate. Even less is required when the animal is fed. The immature castrates seem to become slightly more sensitive as the time from castration increases. This is seen better in the case of theelin. Further division of the dose also tends to increase the potency on the immature normal rat but not to

the same degree. Theelol seems also to be less effective on the ovariectomized animal than on the normal immature animal. The difference is much less striking than in the case of Extract 5 but quite definite. A striking difference would not be expected since the dose of theelol required for a 50 per cent response on the adult castrate is much the same as for the immature normal rat under these conditions of assay.

Transplantation Experiments on Adult and Immature Rats.

In an endeavour to elucidate further the possible role of the ovary in the change of sensitivity observed when the immature rat was ovariectomized, the following type of experiment was devised. The immature female rats were castrated, usually at the age of 21 days. They were tested for their response to either theelin, theelol, or the placental crystals. Five or six days later two ovaries were transplanted into the spleen of these rats. The ovaries were from rats 19 days or 21 days old, which were then used as castrates in their turn. The response to one of the various oestrogenic substances was then tested usually 2 days after

transplantation. A series of experiments on adult castrate rats similarly transplanted with 21 day ovaries was also carried out.

Effect of Transplantation of Ovaries on the Action of Extract 15.

GROUP I.

Test Animal	Age (Dys)	Dose γ	No. of Animals				Percent Response	
			Total	Full Sq.	Epith.	Neg.	Sq.	Sq. and Epith.
Castrated	19							
	23	6.2	11	2	2	7	18	36
	28	10.0	11	4	1	6	36	45
Transplanted:	30							
	34	2.7	11	10	1	0	91	100
	41	1.8	11	7	3	1	63	91

Subsequent History.- Of these, 3 showed spontaneous oestrus one week after the last injection. 2 more became cyclic within 60 days of the last injection. The rest were acyclic. One of the acyclic rats had to be killed and showed on autopsy that the spleen still contained the ovarian graft. The graft had increased in weight to 14 mg. whereas 7 mg. is the normal weight of the 21-day ovaries when implanted. The graft appeared to consist of ordinary immature follicles. The rats were 90 days old when last read and may still become cyclic. That some of the grafts persist for some time is shown by the appearance of cycles some time after operation.

It is to be noted that the animals were not much more sensitive on the second injection given before transplantation. It might be expected that the first injection followed so closely by the second might make the animals more sensitive. That this is so in the case of theelin is shown later.

TABLE XV.

Effect of Extract 15 on Transplanted Rats.

Test Animal	Age Dys.	Dose γ	No. of Animals				Percent Response	
			Total	Full Sq.	Epith.	Neg.	Sq.	Sq. and Epith.
GROUP 2. Castrated	21							
	24	9.5	10	2	3	5	20	50
Transplanted:	27							
	30	2.3	10	7	2	1	70	90
	36	1.56	10	6	1	3	60	70
GROUP 3. Castrated	21							
	23	12.0	10	7	1	2	70	80
Transplanted:	26							
	29	1.35	10	8	0	2	80	80
GROUP 4. Castrated	22							
	50	10.2	15	11	2	2	73	87
(9 X in 48 hrs.)								
	50	10.2	16	16	0	0	100	100
	Effect of Theelol on Transplanted Rats.							
GROUP 1. Castrated	21							
	23	1.62	6	1	0	5	16	16
	32	2.43	12	6	2	4	50	66
GROUP 2. Castrated	23							
	26	2.43	20	0	1	19	0	5
	32	3.24	20	11	2	7	55	65
Transplanted:	35							
	37	1.72	20	15	1	4	75	80

As indicated on page 160, the earlier assays of theelin were disappointing. The commercial product when injected in 3 doses in 12 hours did not show the potency claimed for it. In the later assays of the commercial product, the dose has been divided into 6 doses in 36 hours as for the other substances. The results are given below.

TABLE XVI.

The Bioassay of Theelin (P.D.)

Test Animal	Dose γ	No. of Animals				Percent Response	
		Total	Squam.	Epith.	Neg.	Sq.	Sq.and Epith.
Adult Castrate Injection	2.4	13	1	3	9	7	31
	6.0	24	17	-	7	70	70
	8.0	17	12	2	3	70	82
Immature Normal Injection	4.2	10	0	0	10	0	0
	5.0	10	3	2	5	30	50
	5.4	8	1	1	6	12	24
	6.2	10	7	2	1	70	90

It is not known what weight of crystal the unit of commercial theelin represents. The theelin (P.D.) shows the same order of dosage for the adult rat and the immature normal. The dose for the baby normal is of the same order as that required by Doisy for the immature animal. His dose is 3 units. The adult castrates used on this assay were the same which showed a 95 per cent response on 5.0γ of theelol and a 54 per cent response on 20.5γ of Extract 15. Since crystalline theelin having the expected potency has not been isolated by the author, one cannot be certain that the commercial product is pure and not a mixture of theelin and theelol, or theelol itself.

TABLE XVII.

The Effect of Castration
and Transplantation on Theelin Action.

Test Animal	Age Dys.	Dose Units	No. of Animals Total	Squam.	Epith.	Neg.	%Percent Response Sq.	Sq.and Epith.
GROUP 1. Castrated	20 23	4.0	6	2		4	30	30
GROUP 2. Castrated	19 22 27	4.6 5.4	12 6	1 6		11 0	8 50	8 100
GROUP 3. Castrated	19 21 24	5.4 5.8	9 5	0 2	4 2	5 2	0 55	45 77
GROUP 4. Castrated	21 24	4.8	9	8	0	1	89	89
Transplanted	34 37 43 48	4.8 3.6 1.2	9 9 9	9 9 9	0 0 0	0 0 0	100 100 100	100 100 100
GROUP 5. Normal	23	6.0	22	13	8	1	59	91
Castrated	27 30 30	4.65 4.65	11a 11b	6 11	33 0	2 0	54 100	82 100
Transplanted (11b only)	37 41 41 46 46 52 52	4.65 4.65 3.6 3.6 1.2 1.2	11a 11b 11a 11b 11a 11h	11 11 11 11 11 11	0 0 0 0 0 0	0 0 0 0 0 0	100 100 100 100 100 100	100 100 100 100 100 100
GROUP 6. Castrated	24 26	4.5	32	14	13	5	47	85
Transplanted	28 30	1.8	16	15	1	0	94	100
Not "	30	1.8	14	10	1	3	72	79

Transplantation Experiments on Adult Castrates.

The danger of the occurrence of spontaneous oestrus which is present as the immature rat grows older, is present from the beginning when the adult castrate rat is transplanted with immature ovaries. This danger may be partly controlled by reading the vaginal smear every day so as to detect cycles.

Thirteen old adult castrates, previously among those which responded with a 60 per cent positive response to 20.5γ of Extract 15 crystals, were transplanted with 19 day ovaries. The assay was started three days later. Seven days after the operation, the group showed a 46 per cent response to 3.67γ of placental crystals. The material used throughout these experiments was Extract 15 crystals. Of this group, 2 were cyclic after two months. The rest had been acyclic throughout. This is a response on a much less dose than would have been obtained from these rats before transplantation.

Ten young adult castrate rats which had been castrated at 22 days of age were implanted at 29 days with 21 day ovaries. They were all negative to 6.44γ of Extract 15 at 76 days of age when the dose was given by oral administration. They were 100 per cent positive on 4.2γ of Extract 15. On following their smears it was found that one became cyclic immediately. At 93 days of age, 8 out

of 9 of the acyclic rats were positive on 2.04γ. One of these became cyclic later and one showed a continuous squamous flush. At 113 days of age, the remaining 7 acyclic rats showed only one positive on 2.04γ.

A group of 19 young adult castrates from the same group as the above which had been castrated at 22 days of age were transplanted with 21-day ovaries at 69 days of age. At 76 days they showed 18 out of 19 positive on 5.0γ. At 81 days of age they showed 8 out of 9 positive on 2.1γ and 7 out of 10 positive on 4.8 units of theelin. Of the total 20, 6 became cyclic at this point. At 93 days of age, the remaining acyclic rats, 14 in number, showed 7 positives and 4 epithelials on 2.07γ. They were injected again at 113 days of age and showed 8 out of 14 positive on 2.2γ of Extract 17 crystals.

These results on adult castrates are taken to indicate that the transplanted ovary, even though it does not produce cyclic phenomena, is yet capable of influencing the effective amount of placental crystals which must be injected. The presence of what would from outward appearances be said to be a degenerated graft, is capable of reducing the amount of extract necessary to the same order of weight as that required for the immature normal.

Certain control experiments have been done in connection with the transplantation experiments.

The operative control, that is, cutting the uterus and perhaps removing one ovary but not both, has been mentioned above. It has been shown that such animals are no less sensitive to Extract 15

than the immature normal rat.

Nine baby normals were implanted with adult ovaries in the spleen at the age of 23 days and their cycles were followed. No positive response was seen till the young rats were aged 51 days, when one became cyclic. The age of the rats is now 60 days and 5 are cyclic. This means nothing as far as the persistence of the graft is concerned since they have their own ovaries, but it does show that transplantation of even adult ovaries does not produce spontaneous oestrus within the period covered in most of the transplantation experiments reported above.

Ten immature rats castrated at the age of 19 days were implanted with 19 day ovaries when the recipients were 28 days old. No immediate positive response was noted. The rats are now 85 days old and one is cyclic. It is not known whether this is due to the degeneration of the grafts or whether the rest are still immature. One would expect that the rat would come into spontaneous oestrus at a time determined by its own pituitary rather than by the age of the implanted ovary but if Engle is correct in his theory of interaction between the immature ovary and the anterior pituitary, then the presence of an ovary 9 days younger than the rat in which it is, might be expected to delay puberty. This does not take into account abnormal delays due to the abnormal site of the ovary.

Ten more immature rats castrated at the age of 20 days were transplanted at the age of 27 days with 21 day ovaries. One had spontaneous oestrus at the age of 40 days and has been cyclic since,

one became cyclic at the age of 84 days. The rats are now 86 days old. These results indicate that the implantation of immature ovaries into immature castrates will not cause them to come into spontaneous oestrus within the time of the transplantation experiments reported above. Some at least of the grafts persist for some time.

Another control experiment was performed as follows. Ten normal immature rats were implanted with two extra ovaries at the age of 21 days. The ovaries were also from 21 day old rats. Two days later, they were injected in the usual way with 0.83γ of Extract 15. One showed an epithelial response and the rest were negative. This represents about half an immature rat unit and the transplanted ovaries might be producing say half a unit of oestrin, either because they were being absorbed and were liberating the oestrin which has been shown to be present in them on implantation as mushed tissue into adult castrates, or because of some stimulation of the ovary transplanted into the baby castrate. The work of Martins and Rocha (1931) showing that a castrate immature male parabiosed to a normal immature female would cause the latter to come into oestrus, is taken to indicate that the anterior pituitary becomes hyperactive in the castrate male. If this is true of the female, one might expect that the baby castrates might have some change in their pituitaries such that they would stimulate any ovary transplanted into them to produce oestrin. The absence of spontaneous oestrus in the non-treated grafted animals indicates that sufficient oestrin is not

formed for the production of an oestrous response by the transplanted ovaries. The absence of a decrease in the amount of Extract 15 necessary to produce a positive reaction is also indicative of an absence of liberation of much active material by the ovary of itself apart from any effect it may have on the injected material. The required dose of both Extract 15 and theelin appears to be decreased after the immature rat has been castrated some time without implantation. This is especially marked in the case of theelin (Group 5) where the castrate may require much the same dose as the normal at first, say 4.65 units, but after several injections both castrated and transplanted rats show 100 per cent positive response on 1.2 units. The animals are by this time approaching puberty and one could account for the increased sensitivity of the transplanted rats by supposing a pre-pubertial secretion of oestrin increasing to puberty, this adding itself on to the theelin injected so that less of the latter is needed. This explanation does not apply to the castrate. It is probable that the numerous injections may help to explain this case. However, in Group 6, with only one previous injection which showed 47 per cent response on 4.5γ and with rats only 30 days of age, both implanted and castrated rats showed an increased sensitivity. The former showed a 94 per cent response on 1.8 units and the latter showed 72 per cent response on the same dose; the former were, therefore, more sensitive. The fact that one is not sure that one is dealing with pure theelin and, if Marrian is correct, which form of the

keto-hydroxy form is present, makes interpretations of these results difficult. An attempted explanation of the transplantation experiments will be made in the general discussion.

The Continuous Feeding of Theelin to Immature Normal Animals.

It has been shown by several workers (Wade and Doisy,1931) and (Leonard, Meyer and Hisaw, 1931) that large doses of oestrin given continuously to immature rats, besides producing a continuous squamous flush, have a deleterious effect on the developing ovary. The following experiment on the feeding of minimal amounts of theelin (one unit daily) may be presented here.

Immature normal 21 day old rats were fed with one unit of commercial theelin daily by the usual feeding method. There were 11 rats in all. They were fed from 21-50 days of age, a total period of 29 days; the feeding was then discontinued. Seven showed a continuous squamous flush for the first part of the period of injection.

The continuous flush began to break into a more or less cyclic response when the animals were about 40 days old. Three of the rats were castrated at the age of 32 days. The pairs of ovaries

weighed 6, 6 and 7 mg. respectively. All 3 uteri were in full oestrous condition. The squamous flush continued after castration and ceased to be continuous at the same time as the uncastrated rats. Four of the rats showed continuously negative smears. The age of the group of animals showing continuous squamous smears during the injection at the first oestrus after the cessation of the injections, was 53 days for all 4 normal animals. This was only 3 days after the cessation of the injection at which time all 4 showed negative smears. The second cycle occurred at the age of 58 days for 3 rats and at 63 days for the other animal. Two of the animals were castrated at 54 days of age and the ovaries weighed 21.5 and 26.0 mg., body weight 85 and 87 grams. The uteri were enlarged but not in full oestrus. The two non-castrated rats continued to show regular cycles thereafter. Of the group of 4 showing no response to the dose of theelin used, 2 showed the first spontaneous cycle at 58 days of age, one at 63 days and one at 90 days of age. Two were castrated at 63 days of age and weighed 101 and 129 grams, the ovaries weighed 35.5 and 56.5 mg. respectively. The second cycle of the castrated animal which had shown the first cycle at 58 days did not occur until it was 87 days of age. The number of animals is much too few to draw any conclusions but there is an indication that those rats which show a continuous squamous flush when fed one unit of theelin daily, tend to become cyclic earlier than those which do not so respond. This is reasonable in that the rats which are to mature early will have a much more highly developed accessory genital tract even

before puberty and so will be capable of responding more readily to an oestrogenic substance. It is not possible to say whether the theelin hastens or retards the age at which the rats would normally have matured. The dose for this period does not appear to depress permanently ovarian weights greatly below the normal value for the age.

The Assay of Crystals of Extract 17 from Human Placenta.

Most of this extract was used for a carbon-hydrogen determination. By injection into 19 immature normal animals, 1.65γ gave a 21 per cent positive and 63 per cent epithelial and squamous. On adult castrates a rather large dose was used since the original material from which these crystals were made, showed a high adult-castrate-immature normal ratio. Fourteen adult castrate rats showed a 79 per cent squamous and a 93 per cent epithelial and squamous reaction on 22.1γ.

Miscellaneous Experiments.

When it was found that the dose of theelol and theelin were so high when given in 3 injections in 12 hours, attempts were made to slow up the absorption rate of these substances since more continuous absorption induced by dividing the dose, made the substance much more effective. Only a few animals were used. Five rats were negative on a dose of 3γ of theelol given in two injections in cottonseed oil. Also, when adrenalin was injected with the theelol, 3 animals were negative on 5γ when the dose was given once a day for two days. Six units of theelin gave no response on 4 rats when injected in two doses over two days with adrenalin.

Relation of the Crystals to Emmenin and Theelol and Attempted Interconversion Experiments.

The later assays on crude emmenin show that when injected 6 times in 36 hours to adult castrates, it gives about the same ratio for adult castrate to baby normal dose, as do the crystals.

The idea was expressed by Butenandt and Hildebrandt (1931) that the tri-hydroxy form of oestrin was really inactive and that any activity was due to contamination with the keto-hydroxy form. On this basis the crystals isolated from placenta would be purer tri-hydroxy oestrin than more active preparations. Attempts were made to convert the hypothetical last traces of the keto-hydroxy form into the hydrated form. Various means of hydration were tried. Among them was autoclaving the crystals in acid solution at a temperature of 270°C. for two hours. This was tried on Extract 15. After this treatment, 18.0γ on 4 adult castrate rats gave 3 positives out of 4 rats. The potency on the adult castrate had thus not been destroyed. The possibility that conversion might have occurred to the more active form of tri-hydroxy oestrin (theelol) was rendered improbable by a negative response of 4 adult castrate rats to 4.8γ. The potency of the crystals on the immature rat was not changed. The autoclaving was also done on crude emmenin and no change in relative or absolute potency on the adult castrate or immature normal rat were noticed. However, Dr. Collip found that the emmenin potency was all ether extractable after the autoclaving. This indicated that crystals from that part of the activity of crude placental extract which was ether extractable before autoclaving and which showed a similar physiological potency to emmenin, might be the active principle of emmenin. It has not been possible as yet to isolate the crystals from the ether extract of autoclaved placenta, but similar crystals having similar physical and physiological

properties have been isolated from the emmenin fraction of pregnancy urine after autoclaving. This seems good evidence that the crystals are the active principle of emmenin. Their characteristic physiological property of low potency on the adult castrate as compared to the immature normal animal, was determined in the crude preparation before they were actually isolated.

Another attempt to separate the hypothetical contaminating keto-hydroxy oestrin was made by recrystallizing the substance from hot water several times. It was found possible to get 1.5 mg. of the crystals into solution in 3 cc. of boiling water. This is the amount which separated out on cooling; the amount left in the water when cold is probably quite small. This gives a solubility of 50 mg. per 100 cc. of boiling water. On assay on adult castrates, the material after 4 water recrystallizations gave 3 positives out of 10 rats on a dose of 18.0γ. On the immature normal rat, it gave 2 positives and 8 epithelials on 20 rats, this corresponds to a 10 per cent squamous and a 50 per cent epithelial and squamous response on 1.75γ. There seems to be a slight decrease in potency in both groups, but with the variation seen before in immature rats and with the small number of castrates, nothing definite can be said. The potency on the castrate is certainly not entirely lost.

Effects of Light on the Hormone.

Jordan and Doisy (1926) have shown that light inactivates the crude active material. Allen and Ellis (1925) and Ludwig and Reis (1931) showed that ultraviolet light was especially powerful in this respect. The latter authors claim that red light, on the other hand, increases the activity of the substance and reverses the inactivation previously caused by ultra-violet light. They worked with crude progynon and exposed the material to the light for two hours at a distance of 60 cm. Irradiation of theelin, theelol and the placental crystals with a carbon arc lamp of range about 1850-10,000 Å, was carried out. Eosin was added to some of the material because of Jordan and Doisy's suggestion that it sensitized the hormone to light. No definite effects have yet been observed. The longest period of irradiation has been an hour. The solution of the material in water is placed in an open vessel with the mouth towards the carbon arc at a distance of about 6 inches. No filter was employed. Two rats, positive the previous week on 1.5γ theelol, were negative on 15 units of theelin after irradiation with eosin. Theelol and Extract 15 did not seem to alter in potency. The author hopes to carry out further experiments along this line. From the absorption curve of Butenandt, it would seem that light of 2850 Å or 2100 Å could be most effective in inactivation.

DISCUSSION

Preparation of the Crystalline Substance from Placenta.

Marrian's method seems to work quite satisfactorily. No doubt, modifications could be introduced which would improve the recovery and shorten the method. It is much more difficult to obtain crystalline material from placental extracts than from those of pregnancy urine. The adjustment of the pH to neutral or faintly alkaline reaction is probably helpful before the chilling with ether. The use of CO_2-snow and acetone for chilling and the addition and evaporation of several aliquots of ether at this point, also seem to aid crystallization. It is probable that an increased proportion of acetone in the original placental extraction would improve the yields. The extraction at some time from sodium bicarbonate solution seems to be important since those extracts where this was not done were usually failures as far as isolation of crystals was concerned.

The Nature of the Crystalline Substance.

The closeness of the melting point and the absence of

lowering of melting point when mixed with theelol indicates that the crystals are closely related to the tri-hydroxy form of oestrin. The higher hydrogen content in the carbon-hydrogen determination requires further confirmation. Marrian reports that interference with the phenolic hydroxyl by formation of the mono-methyl ether inactivates the tri-hydroxy form of oestrin. This indicates the possibility of a change at this point though it is difficult to see what change is possible. It is difficult to believe that saturation of a double bond could occur without change in melting point.

In contrast to this close chemical relationship stands the relative inactivity of crystalline emmenin on the adult castrate, as compared to that of theelol. The property of difference of physiological effect with apparent identity of gross chemical structure occurs also in the inactive form of tri-hydroxy oestrin isolated by Butenandt from human pregnancy urine and apparently also by Dohrn and others from the urine of pregnant mares. It is interesting in the last instance to compare the lack of ether solubility characteristic of emmenin in the crude state, with the similar ether insolubility pointed out by Zondek for activity in mare's urine. The isolation of a comparatively inert substance, first by the author from human placenta and subsequently by Dohrn and others from mare's urine, suggests some connection between the two properties. The Science News Letter for April 16, 1932, carries a report that two new sex hormones containing the same number of carbon atoms (18) and oxygen atoms (2), but two less hydrogen atoms (20) than theelin, have been isolated by Girard and Sandulesco from the urine of pregnant

mares. The substances are said to differ from each other only in the arrangement of the molecule. The substances are said to act only on one of the accessory sex organs which is not stated. They call their hormones 'equiline' and 'hippuline'. No further details of this work are available at the present time. If true, it increases further the number of substances having sex hormone-like activity. This activity will probably soon be attributed by many to a whole class of substances having a certain type of chemical configuration in common as was formerly said in the case of vitamin A. If the decreased number of hydrogen atoms is correct, then the possible presence of two more hydrogen atoms than the usual number for tri-hydroxy oestrin in our own compound, may make the number of hydrogen atoms in the compounds of this type of significance for their physiological potency. However, the ether insolubility may be due merely to a salt formation with some organic acid.

The water solubility of the placental crystals is higher than that reported for the keto-hydroxy form but no figures are available as to the solubility of the tri-hydroxy form in pure water. It is merely said to be more soluble. As mentioned above, Butenandt suggests two reasons for the relative inactivity of his product. Either that the tri-hydroxy form is inert and that the activity is due to contamination with the keto-hydroxy form or that the substance is an isomer of active tri-hydroxy oestrin.

The contamination theory requires that the percentage of contamination remain remarkably constant since Marrian and Doisy

consistently get products which have identical potencies as compared with their other products. The fact that recrystallization does not change the potency is not conclusive evidence that no mixed crystal formation occurs. All attempts at changing the potency of crystalline emmenin have failed. It is possible that the difference lies in the original source of material and that the substance in placenta differs from the first form that is excreted in the urine.

The inability to obtain a completely inactive product may have an explanation other than the contamination with the keto-hydroxy form. The tri-hydroxy form may be completely inactive in itself but be converted in the body of the castrate animal to the active form. The fact that dividing the dose increases the efficiency of the active substances so greatly may not be due to excretion or destruction wholly but also to a greater efficiency of conversion into an active form. It is not known which of the forms of oestrin exists as such in the body. If one regards the most highly active form of theelin (Marrian's A form) as this substance, then it is true that Butenandt found that dividing the dose from one to six injections increased the potency five times, so that all the increased potency cannot be due to conversion to this substance. The same division of the dose of Marrian's tri-hydroxy form increased its potency on an average seven times. The increase for Butenandt's active product on dividing the dose is not given. It is difficult to compare our method of assay with his. He gives the

potency of his preparation as 100,000-200,000 mouse units per gram for a single injection in oil to castrate mice. Our material has a potency of 60,000 units per gram in 6 doses over 36 hours in castrate rats. This is 675 times less potent than the most active preparation of the keto-hydroxy form tested on the castrate mouse in the same division of doses.

The results of Marrian on the relative time required for the greatest number of positives to appear after the last injection is of interest in connection with the theory of conversion. For his active tri-hydroxy product, he finds the maximum at 24 hours after the last injection and a sharp fall to no positives in the next 24 hours. For the B form of keto-hydroxy, the response is slightly prolonged and the peak is at 34 hours. For the A form of this substance, the latent period is 48 hours before a maximum of positives, a second prolonged maximum response occurs at a much later period. It is difficult to regard the tri-hydroxy form as being converted into the keto-hydroxy form before becoming active since it acts more quickly than the form into which it is supposed to be converted. The picture is complicated by the greater water solubility and hence, probably greater rate of excretion of the tri-hydroxy form. The prolonged maximum of the active form of keto-hydroxy oestrin is also very difficult to explain. We are able to confirm Marrian as regards the time of maximum response. Both with the placental crystals and with theelol on the 100 adult castrates, the point of maximum number of animals positive was about 24 hours after the last injection

and the response fell off rapidly so that no animals were positive at the end of 48 hours after the last injection.

Though it is not probable that the actual conversion is one of tri-hydroxy to keto-hydroxy, we feel that some form of the conversion theory may be used to explain many of the experiments in this investigation. The fact that division of the dose tends to obliterate the ratio between the adult castrate or immature castrate and the immature normal, speaks for better utilization of the substance on the part of the immature normal since it requires less division of dosage for a given weight of substance to be effective. The comparison of the effect on the immature castrate and the immature normal is more useful than that between the adult castrate and the normal animal, because of possible differences in absorption and excretion rate in the adult castrate and immature normal animal and also because of weight differences. When the effect of the emmenin crystals on these two types of animal are compared, a definite difference of physiological potency is seen. The crystals are about 8 times more active on the immature normal animal than on the just castrated immature animal. The comparison must be made with this particular division of the dose since further division results in a disturbance of the ratio. It is felt that this greater potency on the rat with the ovary intact, indicates that the ovary immature or not acts on the placental crystals to make them more readily utilizable to the animal. It is not possible to say to what form the crystals are converted but we believe that the ovary does

change them to some other more active form.

Doisy finds that theelin is 3 times as effective on the adult castrate as it is in the immature normal. We have not used pure theelin but commercial theelin which may not be pure. The weight for a unit on our adult castrates is slightly more than for the immature animal. For theelol the unit for the adult castrate is approximately the same as for the baby normal; that for the immature castrate seems to be slightly greater according to our method of assay. The ratio of pure theelin, both A and B form, will have to be done before one can make any statement as to the mechanism of action of the ovary on these compounds and into what form it may convert them.

The transplantation experiments also indicate that the ovary has an action on the placental crystals. The transplanted animals show about the same potency for these crystals as do the immature normals.

One obvious explanation for these results is that as mentioned above, the ovary contributes part of the necessary active substance from its own oestrin, thus rendering the amount which has to be injected smaller. The experiment on the implantation of extra ovaries into the normal immature animal and the proof that half the usual amount of the placental crystals will not cause a positive response, is against the contribution by the ovary itself. However, under the conditions of prior castration of the animal according to the work of Martins and Rocha (1931), the pituitary might be expected

to stimulate the ovary transplanted into previously castrated animals, to produce more oestrin than ovaries transplanted into animals with their own ovaries still present. The amount of oestrin so produced must be under the amount necessary for an oestrus response in the case of our experiments since transplantation alone never produced a positive response. The time between castration and implantation in our immature animals was usually quite short so that pituitary changes should not have time to occur. The fact that both castrated and implanted animals show a decreased sensitivity to commercial theelin as compared to the sensitivity to a dose given some days previously to the same group when all the animals were castrated complicates the picture further. Until pure theelin can be tried on the castrated animal and the animal with a transplanted ovary, one cannot say definitely whether the transplanted ovaries contribute any active substance from their own store of oestrin or whether they act only by converting the injected material into a more active form. The fact remains that the presence of an ovary makes a great difference to the amount of emmenin crystals needed for a positive response or very little to the amount of theelol needed or theelin.

The variations in response of different groups of immature normal animals is considerable. This may be correlated with a difference in development of the accessory genital tract making it less sensitive in some animals because it is less developed. On the theory of conversion by the ovary the variation in sensitivity may be

correlated with differences in degree of development of the ovary itself and hence of its efficiency in performing the conversion process. That ovaries do differ greatly in degree of development in the immature animal is well known. The experiments on continuous feeding of theelin seem to indicate that animals which mature early respond better to theelin before puberty than those which mature late. The relative importance of the ovary or of the development of the accessory organs under the influence of the ovary in producing this effect is unknown. The experiments on adult castrates where the transplantation of immature ovaries did not cause spontaneous oestrus but did decrease the dose of placental crystals from the adult castrate unit to approximately the immature normal unit are to be noted. This increased sensitivity apparently persisted for some time. Most of the animals remained acyclic for the whole of this period. After about 6 weeks, in some animals there was indication of decreased sensitivity. The dose was not raised to near the adult castrate unit so that the degree of return to the castrate condition is unknown. Two possible mechanisms for this effect are the following, the possible action of the transplanted ovaries in secreting enough oestrin to cause a 'tonic' effect on the accessory sex organs thus making them respond more readily to the placental material, and their other possible action in converting the placental material into an active form. It is felt that the positive response of an acyclic adult animal to an injection of crystalline emmenin in a dosage equal to that to which the immature normal rat responds, is

an indication of the presence of functional ovarian tissue in the animal even though the amount or character of this tissue is such as not to produce oestrous cycles. On this view, the hypophysectomized rat should respond to the immature rat dose of emmenin. Provided, of course, that the picture is not complicated by the presence of the corpus luteum hormone from functional corpora lutea in the ovarian tissue.

The part of the ovary responsible for the conversion action is not known. Mature follicles are not necessary and an histological study of grafted ovaries should show whether the response to the immature rat dose continues when all follicular elements have disappeared.

Doisy is able to use much less of his crystalline theelol to obtain a good response than has been found necessary in this laboratory. He also is able to obtain this potency on giving 3 injections in 12 hours, whereas we have found that most materials are much less sensitive by this method. It is felt that part of the explanation lies in the selection of the animals. Doisy's unit is defined by means of a closed circuit so to speak, since he selects his rats so that they will not respond to 0.75 units and will respond to 1.25 units. Thus his unit might be defined as the amount of material necessary to bring 75 per cent of animals which will respond to one unit of oestrin into oestrus. This is justifiable as long as a standard preparation of uniform potency is available. One could not detect differences in potency of crystalline material in this way.

Apparently it is possible in the field of sex hormones to have superficial chemical identity with quite different physiological potency. If an apparently pure crystalline substance showing the same melting point etc. as a previous preparation were to give a negative response, the animals would be discarded as insensitive. If this process of selection is carried on over a period of years it is possible to obtain a group of hypersensitive animals. It is felt in any case that the use of a large number of unselected animals gives a truer picture of the physiological action of any substance upon the rat population as a whole.

The Significance of the Isolation of the Crystalline Material from Human Placenta.

The discovery of an oestrogenic substance relatively inert on the adult castrate raises the whole question of the distribution of oestrogenic substances in nature. It is of much more physiological significance to know the distribution of substances which can be utilized by and will act on the animal when its ovary is present than those which act only when it is castrated. The castrate is an artificially produced and abnormal animal and as a test object can have significance only for other castrates. The distribution of

oestrogenic substances in nature should it is felt be reinvestigated using the immature rat as a test object.

There are practical difficulties in connection with the assay of materials on the immature rat since each animal can be used only once and this necessitates a large rat colony. The individual variations found in immature rats just as in adult rats necessitate the use of large numbers of animals on each dose. The variation in sensitivity tends to run parallel in litter mate sisters, whole litters being sensitive or insensitive. For this reason, in an assay it is well to select from several litters rather than taking all the animals from one large litter.

The significance of the presence of emmenin in the placenta and the relation of its physiological properties to its function during pregnancy is not known. Emmenin as a non ether-extractable compound has not been demonstrated in placenta other than human. The cow, the pig and the rat placenta show no such fraction. The importance for the use of the hormone in medicine of the use of the immature animal as a test object, is considerable. Marrian and Parkes using an extract in which considerable material, active on the immature normal rat but inactive on the adult castrate, might exist have calculated that fantastic doses are necessary for any clinical effect. Once again the adult castrate is an unsuitable test object since most patients to which the hormone is administered are not operative castrates and it has been shown in the case of the adult castrates implanted with immature ovaries, that even an ovary

which is not capable of producing cyclic changes is still able to render the animal sensitive to the emmenin crystals in an amount of the order of the immature rat unit. There is, of course, no reason to suppose that the human would react in a comparable dose on a weight basis to that to which the immature rat responds; but it is felt that the animal with an ovary, even though an immature one, is more comparable in its reactions to the human being with an ovary even though a poorly functioning one, than to the castrate rat.

SUMMARY.

1. A crystalline oestrogenic substance has been isolated for the first time from placenta as the source material. The human placenta was used.

2. The melting point of this substance is 274°C. (uncorrected). Earlier melting points ran from 259°C. to 268°C. and were indefinite. A carbon-hydrogen determination on one of the preparations gave a value of carbon 74.8 per cent and hydrogen 8.8 per cent.

3. The crystal form of various preparations has varied. The crystals are monoclinic, optically positive, have refractive indices of α 1.54, β and γ 1.68. The crystals show an inclined extinction of 7°.

4. The solubility of the crystals in neutral aqueous solution at boiling temperature is about 0.05 per cent.

5. A mixed melting point of the compound with a sample of crystalline theelol (kindly supplied by Dr. Doisy) gave no significant lowering of the melting point. Theelol melted at 276°C., the placental crystals at 274°C. and the mixture at 275°C. (all uncorrected).

6. The physiological potency of the placental crystals when assayed on the adult ovariectomized albino rat shows a weight of 15.75γ necessary for a positive response (as measured by the vaginal smear method requiring full squamous cornification for

a positive response) in 62 out of the 100 animals injected. This potency is obtained when the dose is given in 6 subcutaneous injections over 36 hours. When given in 3 doses over 12 hours, the substance was inactive up to 72γ on the adult ovariectomized rat.

7. This potency is much lower than that of crystalline theelol on the same 100 rats. A dose of 1.57γ was sufficient to cause a positive reaction in 80 out of the 100 adult rats used. The division of the dose was the same as that in the case of the placental crystals.

8. The physiological potency of the placental crystals on the immature normal albino rat of 21 days of age is much greater than that on the adult castrate. The weight of crystals required for a 47 per cent positive response in a group of 30 immature normal rats was 1.55γ. The sensitivity of the immature rat varies considerably. Whole sensitive or insensitive litters are sometimes encountered.

9. The weight of theelol for a 45 per cent response on 9 animals was 1.62γ or the same as that of the placental crystals for a similar response on the immature normal rat.

10. The effective oral dose when administered in the drinking water of the animal over a period of 3 days was approximately the same as that required for injection on the immature rat. This applied to both the placental material and to theelol.

11. The dose of the placental crystals required for the immature ovariectomized animal spayed at 21 days of age two to four days

after castration, was much greater than that for the immature normal rat of the same age. 10.0γ of the material gave a response of 4 rats out of 11 positive or 36 per cent. The dose of theelol for the same type of test object was also slightly higher than that required for the immature normal rat but the increase was not nearly as great as in the case of the placental material. 3.24γ of theelol caused a 55 per cent response when injected into 20 immature castrate rats.

12. These results indicate a definite difference in physiological effect between the crystals obtained from placenta and theelol.

13. The ratio between the dose required for the immature normal and immature ovarectomized rat tends to disappear on division of the dose into a large number of parts or on oral administration.

14. Transplantation of 21 day ovaries into the spleen of immature castrate rats at the age of 26-27 days causes the dose of placental crystals necessary for a given response to fall from the immature castrate level to the dose needed for an immature normal rat of 21 days of age.

15. Transplantation of immature ovaries (19 or 21 day) into adult castrate animals, though it did not often cause the appearance of cycles, always caused the reduction of the dose of the placental crystals from that necessary for the adult ovariectomized rat to or near to the dose needed for the immature normal rat. This increased sensitivity persisted for

at least six weeks during which time only a very few of the rats showed spontaneous oestrous cycles.

16. The transplantation of adult ovaries into immature normal rats or of immature ovaries into immature ovariectomized rats does not cause oestrus to occur before the normal age of puberty.

17. Implantation of the two extra ovaries into the immature normal spleen does not cause the animal to need less of the placental crystals than the ordinary immature normal rat to bring it into vaginal oestrus.

18. No definite effects on the growth of immature rat ovaries have been observed with the placental crystals.

19. Commercial theelin appears to act satisfactorily in a dose of about 6 of the units marked. Castration of the immature animal seems to increase the sensitivity of the rat to commercial theelin.

20. The continuous feeding of 1 unit of commercial theelin daily to immature normal rats causes the appearance of a continuous squamous flush in some of the animals. There are some indications that those rats which respond with a continuous squamous reaction to this dose of theelin are the animals which mature early and that the non-responsive ones mature later.

21. Evidence is presented for the identity of the crystals from placenta with the hormone called emmenin previously demonstrated in the crude form in human placenta by Dr. Collip.

22. Attempts to alter the physiological potency of the crystals have not been successful.

23. The ovary is considered to play an important role in the difference in potency in the adult castrate and the immature normal animal.

24. A theory of the mechanism of action of the ovary in this connection is presented.

25. The importance of the immature rat as a test object is discussed.

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