# "THE DISTRIBUTION OF PROGESTERONE IN BODY FLUIDS AND TISSUES OF THE DAIRY COW"

by

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## A C K N O W L E D G E M E N T S.

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SECTION 4.

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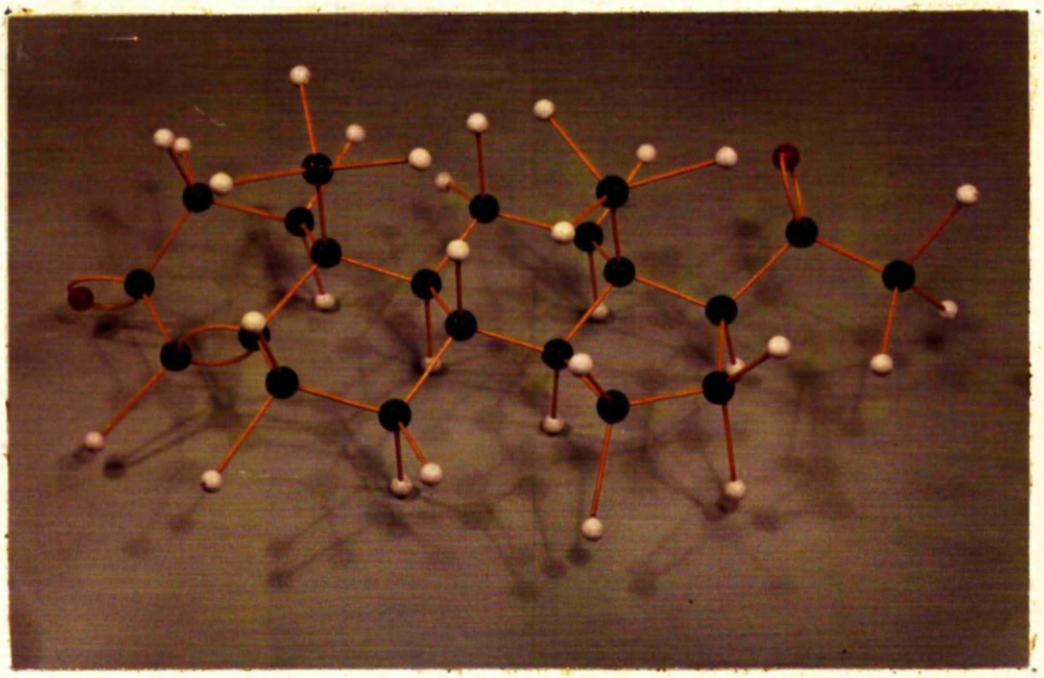
FIGURE 1.A.1.



SPATIAL MODEL OF PROGESTERONE

(PREGN - 4 - EN - 3:20 - DIONE)

BLACK = CARBON

RED = OXYGEN

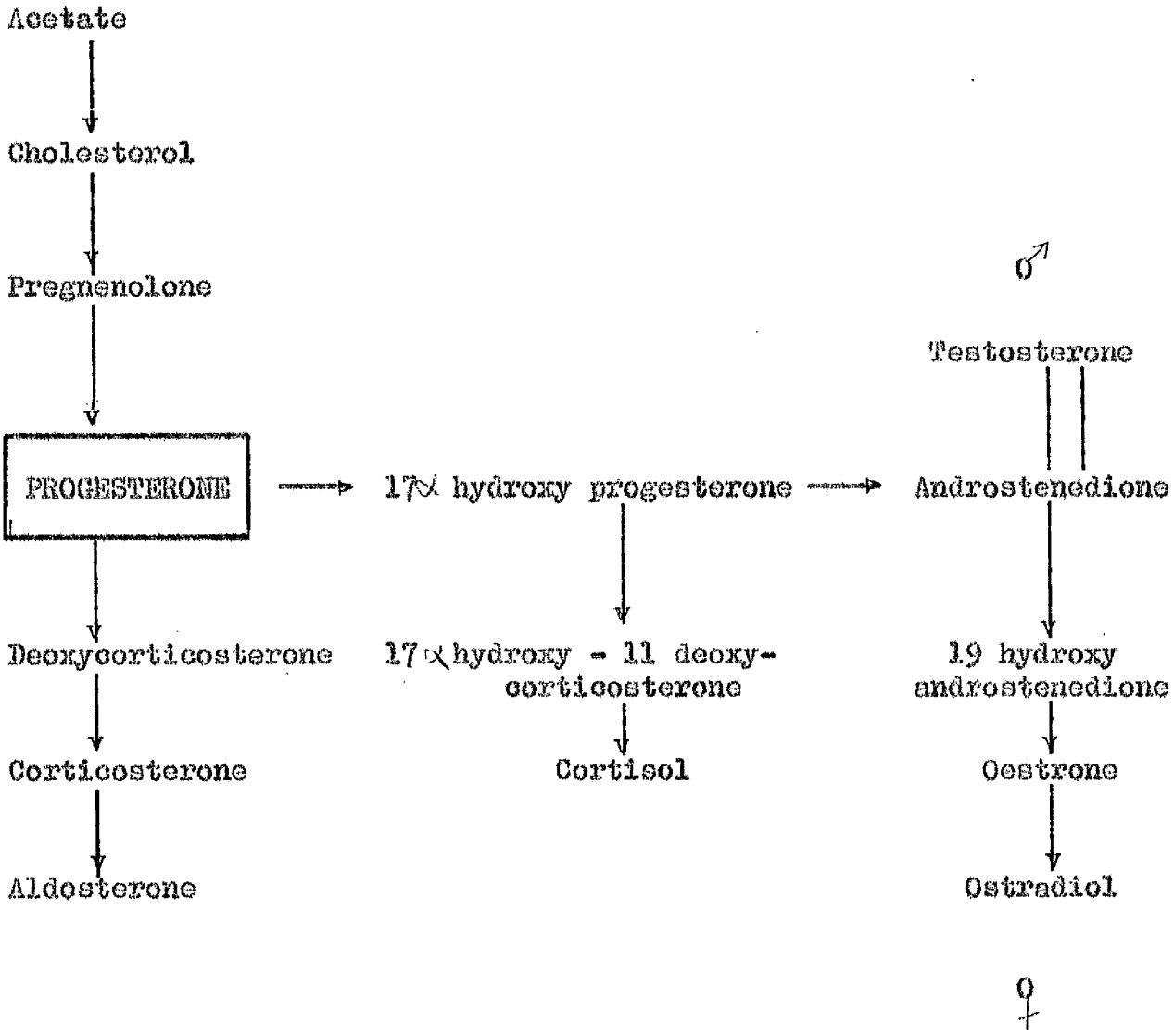
WHITE = HYDROGEN

$C_{21} H_{30} O_2$

FIGURE 1.A.2.

PROGESTERONE IN THE STEROID BIOSYNTHETIC PATHWAY

Diagramatic after Symington (1959); Goodwin (1960); Short (1960) and Grant (1962).



# SECTION I - INTRODUCTION.

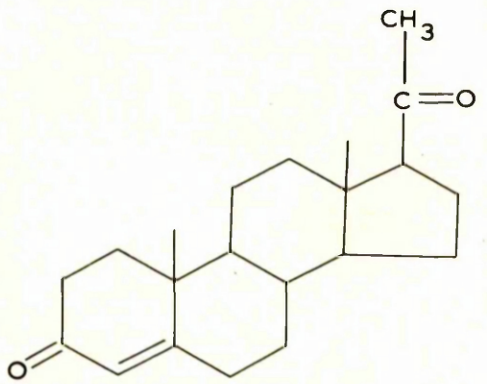
## A. PROGESTERONE IN THE ANIMAL KINGDOM.

Progesterone is the hormone responsible for the maintenance of pregnancy in mammals. The main sources of this hormone are two temporary structures, the corpus luteum and the placenta. Progesterone is also found in the adrenal gland and in the ovarian follicular fluid prior to ovulation.

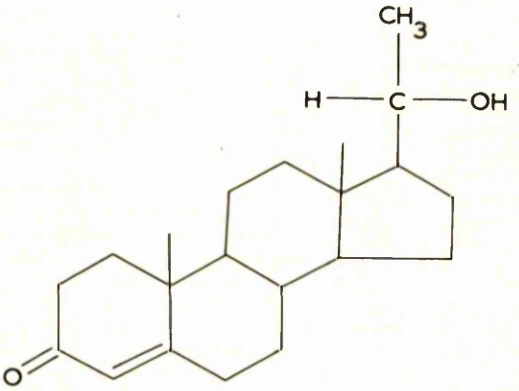
Progesterone is an important intermediary in the biosynthesis of the majority of other steroid hormones. (Figure 1.A.2.). Its presence in follicular fluid is indicative of its biosynthetic role and also a possible role in the mechanism of ovulation. (Catchpole, 1959; Forbes, 1953).

During pregnancy progesterone is responsible for the provision of a uterine environment suitable for the growth and development of the foetus. Also at this time, in most species of mammal, it is responsible for the reduction of cyclic ovarian activity, and partly responsible for mammary development.

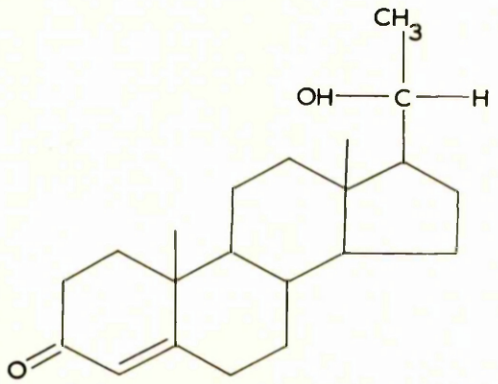
**FIGURE 1.A.3.**



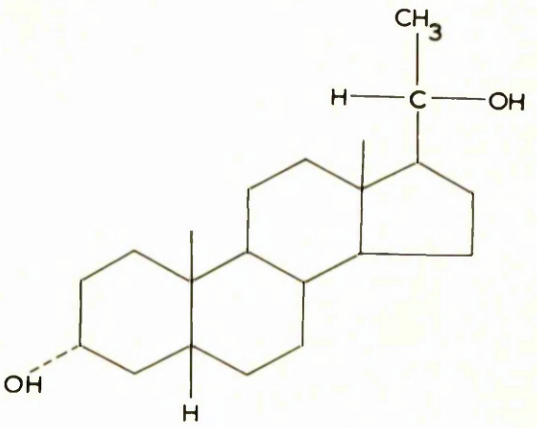

PROGESTERONE
(PREGN-4-ENE-3:20-DIONE)




20α-HYDROXYPREGN-4-EN-3-ONE




20β-HYDROXYPREGN-4-EN-3-ONE




PREGNANEDIOL
(5β-PREGNANE-3α:20α-DIOL)

Among the more unusual biological effects are the thermogenic and anaesthetic properties of progesterone. Evidence of the former is seen in the rise in body temperature of women following ovulation (Tompkins, 1945). This phenomenon is not seen in the cow after ovulation but can be demonstrated following parenteral administration of progesterone (Wrenn, Bitman and Sykes, 1959). In common with a number of other steroids, progesterone, when injected intravenously, has the property of a short-acting general anaesthetic. (Selye, 1941; and Merryman, Boiman, Barnes and Rothchild, 1954).

Progesterone is also found in the lower animals where its exact function has not yet been elucidated.

The structural formulae of progesterone and related compounds which are mentioned in the text are shown in Figure 1.A.3. See also Figure 1.A.1.

## B. HISTORICAL SURVEY.

Volcherus Coiter (1573) described the presence of cavities filled with a yellow solid in the ovary. de Graaf (1672) gave a more definite account of these structures and noticed that their number appeared to be related to the number of foetuses in utero and therefore assumed that they only occurred after mating.

Malpighi (1689) using his microscopical techniques gave the first accurate account of the structures and applied the name 'corpus luteum'. Beard (1897) was the first person to put forward a suggestion of the corpus luteum function which has survived the test of time. He postulated that it was responsible for the suppression of ovulation and oestrus during pregnancy. About this time Prenant (1898) suggested that it might be a gland of internal secretion directly benefitting the "egg" with which it was associated.

It was not until 1903 that the function of the corpus luteum in pregnancy was conclusively proved by Fraenkel (1903) who demonstrated the necessity of the corpus luteum in the rabbit for the implantation of ova and the

subsequent maintenance of pregnancy. In 1908, Loeb demonstrated the requirements of the corpus luteum in the ovary for deciduoma formation in the guinea pig.

Loeb and Hesselberg (1917) found that removal of the corpora lutea in pregnant guinea pigs allowed ovulation to take place. In addition they were able to shorten the cycle length in unmated guinea pigs from which the corpora lutea were removed shortly after their formation. This finding was subsequently confirmed in the cow by Williams (1921) and Hammond (1927) who were able to shorten the 21 day cycle in the cow by manual expression of the corpus luteum at mid cycle; oestrus and ovulation following in 2 to 5 days.

Loeb in 1923 reported that follicles may develop in the guinea pig in the presence of the corpora lutea but that ovulation did not occur. Hammond (1927) reported a similar situation in the cow.

Corner and Hurni (1918) injected rats with aqueous extracts of sow corpora lutea but were unable to delay ovulation or alter the cycle.

Kennedy (1925) repeated this experiment in rabbits

and although ovulation was inhibited in some subjects he was unable to come to any conclusion due mainly to the toxic effect of the extract.

Papanicolaou (1926) prepared a lipid extract of sow corpora lutea and, using weekly injections into guinea pigs, he was able to suppress oestrus and ovulation as long as injections were continued.

Corner (1928) and Corner and Allen (1929) were able to produce endometrial proliferation in rabbits by injecting material obtained from sow corpora lutea by hot alcohol extraction. (Substances which produced this change were known as "progestins"). From this endometrial response they were able to devise a simple dose/response assay for "progestins". A major development followed the above experiments when the same authors using their extracts of sow corpora lutea were able to maintain pregnancy in ovariectomised female rabbits. (Allen and Corner, 1929).

Allen, in 1932, prepared a fairly pure form of this pregnancy "compound", while Fevold, Hisaw and Leonard (1932) also prepared a substance of high activity from sow corpora lutea. A great deal of interest was taken in these

experiments and soon afterwards in 1934, the isolation of pure crystalline hormone extracts from corpora lutea was announced simultaneously by four groups of workers (Butenandt, Westphal and Hohlweg, 1934; Hartmann and Wettstein, 1934; Slotta, Ruschig and Fels, 1934a and 1934b; and Wintersteiner and Allen, 1934). Of the four groups of workers it was Slotta et al. who designated the compound progesterone and suggested a structural formula. Soon after this Butenandt and Schmidt (1934) synthesised progesterone from pregnanediol which had been isolated from pregnant women's urine by Marrian (1929). This synthesis confirmed the prediction of Slotta et al. (1934) and demonstrated for the first time a relationship of pregnanediol to the newly isolated hormone of pregnancy, progesterone.

## C. URINARY PREGNANEDIOL AS AN INDEX OF PROGESTERONE METABOLISM.

Following the realisation that pregnanediol was probably the main metabolite of the newly discovered pregnancy hormone, progesterone, Venning and Browne (1936) and Venning (1937) described a method for the gravimetric determination of pregnanediol in the form of sodium pregnanediol glucuronidate. The method suffered from the defect that the final extract was not pure and that spontaneous decomposition of sodium pregnanediol glucuronidate occurred in the urine. In spite of these shortcomings they were able to demonstrate the physiological relationship between pregnanediol and progesterone by isolating the former compound from the urine of subjects who had received progesterone by injection. This was followed by a report on the excretion of pregnanediol as an index of progesterone metabolism in women (Venning and Browne, 1937).

Astwood and Jones (1941) eliminated some of the defects of the above method by introducing an acid hydrolysis of the urine. The final product was pregnanediol which was estimated gravimetrically. The same year Talbot, Berman,

MacLachlan and Wolfe (1941) developed a procedure for estimating free pregnanediol colorimetrically as the sulphuric acid chromogen.

Several improvements of the "Astwood-Talbot" method were elaborated by different groups of workers. Enzyme hydrolysis was introduced by Talbot, Ryan and Wolfe (1943) and later by Cohen (1951). This avoided the formation of the unwanted pigments produced by acid hydrolysis.

Slow cooling to effect the precipitation of pregnanediol was one of the improvements advocated by Sommerville, Gough and Marrian (1948).

Chromatography for preliminary purification was utilised by a number of people (Huber, 1947; de Watteville, 1950; Stimmel, Randolph and Conn, 1952, and Chaney, McKee, Fischer and McColgan, 1952). It subsequently became apparent that these latter methods suffered from the disadvantage that impurities were giving rise to an over-estimate in the final readings.

Klopper, Mitchie and Brown (1955) and Klopper (1956) devised a relatively simple but specific method for pregnanediol suitable for routine laboratory use. This involved

the use of column absorption chromatography and subsequent acetylation for further purification. Final estimation was based on the sodium sulphite/sulphuric acid chromogen of the di-acetate. Although acid hydrolysis was used in this method there appeared to be no unwanted pigments interfering with the final estimation.

Pregnanediol was reported to be present in large quantities in the urine from pregnant cows (Marker, 1938) but O'Moore (1947) was unable to substantiate this finding. A number of different groups attempted to settle this anomaly using a variety of the methods described above. (Cowie and Greenbaum, 1948; Stevenson, 1947; and Hill, Peterson and Cohen, 1954). However, none of these studies revealed the presence of pregnanediol in sufficient quantity to warrant any kind of quantitative estimation. In 1959, Klyne and Wright, in a very careful chemical study of the urinary steroids of the cow, were able to isolate only trace amounts of pregnanediol in addition to a variety of other "diols". From this and the report by Wright (1958) it would appear that the most important urinary metabolite of progesterone in the cow is 5β-Androstane-3α :17α diol which is found in very small quantities in the urine. A possible explanation

of the high levels of pregnanediol found by Marker (1938) in cows urine is given by Wright (1958) who suggested that faecal contamination of the urine may have occurred, particularly in view of the fact that a number of C21 diols have been isolated from cow bile (Pearlman and Cerceo, 1948).

It is apparent that in animals other than man the route of excretion of progesterone metabolites is via the alimentary tract, as it has been shown that injected progesterone - 4 - C14 results in a high percentage of biliary excretion of the radioactivity in cats (Taylor and Scratchard, 1960) and faecal excretion in rats (Shen, Elliot, Doisy and Doisy, 1954).

In addition faecal androgen excretion in the cow is increased following the intra muscular injection of progesterone (Miller and Turner, 1955). It would therefore appear that pregnanediol is of no value as an index of progesterone metabolism in the cow.

The alternative methods available for the study of progesterone metabolism in the cow are:

(A) Bio-assay Methods and

(B) Chemical Methods.

These will be discussed under separate headings.

## D. SURVEY OF THE BIO-ASSAY METHODS USED FOR PROGESTERONE ESTIMATION.

Using the method of Corner (1928) and Corner and Allen (1929) in which alcoholic extracts of luteal tissue were injected into ovariectomised rabbits, Kimura and Cornwell (1938) measured "progestins" in sow corpora lutea during pregnancy and the oestrous cycle; the response being measured subjectively as the degree of endometrial proliferation. Using similar criteria, Bloch (1936) measured "progestins" in peripheral blood. The levels he found in the pregnant sow were, however, shown to be a considerable over-estimate. (Short, 1958[a]).

A variation of the Corner and Allen method was described by Clauberg (1930) utilising oestrogen treated immature female rabbits in which ovariectomy was unnecessary. McGinty, Anderson and McCullough (1939) increased the sensitivity of the latter method by introducing the extract into an isolated segment of the uterine horn and noting the decidual response. This method was used in a number of studies in an attempt to measure "progestins" in plasma of different species. (Haskins, 1939; Haskins, 1941; and Hoffman and Von Lam, 1948).

Shapiro (1936) evolved a test for "progestin" in which he injected extracts into the toad (Xenopus laevis). A positive result was indicated by ovulation and oviposition. However, this was very non-specific as a large number of other steroids, and also gonadotrophins, gave a positive result.

Duyvené de Wit (1938, 1941) developed a bio-assay for progesterone using the female bitterling (Rhodeus amarus) in which the response to the extract was measured by the behaviour of the ovipositor. He obtained some indication that progesterone was present in the blood of the cow and the pig.

Hooker and Forbes (1947) devised a method of progesterone assay which had a sensitivity of 0.3 micrograms per microlitre. This involved the introduction of an extract into an isolated uterine segment of the ovariectomised mouse followed by an assessment of the hypertrophy produced in the 'stromal nuclei' of the endometrium.

Several groups employed this method for the measurement of peripheral blood "progestins" in a number of different species:

Forbes, Hooker and Pfeiffer (1950) - Monkey.

Forbes (1951) - Human and Monkey.

Bryans (1951) - Monkey.

Meher and Zarrow (1954) - Ewe.

Zarrow and Meher (1955) - Rabbit.

Generally speaking the results were somewhat variable but many bore a relationship to subsequent chemical determinations of progesterone.

Bio-assay methods however suffer from the defect of being relatively non-specific for progesterone and efforts were continuously being made to develop a specific chemical method for the measurement of progesterone.

## E. CHEMICAL METHODS FOR THE ESTIMATION OF PROGESTERONE.

Properties of progesterone: Progesterone (pregnene-4-en-3:20 dione) is a 21 carbon steroid hormone possessing a double bond between carbon 4 and 5 and ketone groups at carbons 3 and 20. (See Figure 1.A.3.). In pure form it is a white crystalline solid with a molecular weight of 314 and is very soluble in lipid solvents and plasma but almost insoluble in water. It has, in common with many other $\Delta 4 - 3$ ketone steroids, a well defined absorption peak at 241 mμ in ethanolic solution.

The chemical reactions of progesterone are again shared by a large number of different steroids. These include the Zimmerman reaction, in which progesterone shows up as a blue spot on paper after treatment with alcoholic potassium hydroxide, and the sodium hydroxide fluorescence reaction described by Bush (1952). When treated with sulphuric acid it gives a chromogen with an absorption peak at 290 mμ a feature shared with several other steroids (Zaffaroni, 1950).

Progesterone can be partly reduced with sodium borohydride to yield small amounts of both its 20 hydroxy epimers (Norymberski and Woods, 1955). However, since progesterone has no hydroxyl groups it cannot form an acetate.

Due to the presence of the ketone groups, progesterone will form derivatives with a number of compounds. These include the isonicotinic acid hydrazine derivative (Ercoli, Giuiseppe and Ruggieri, 1952). The bisthiosemicarbazone derivatives (Evans and Gillam, 1943) and the bisdinitrophenylhydrazine reaction (Klein, Weiner and Gordon, 1948).

None of the properties of progesterone so far described are suitable criteria of identification. They can, however, be used as a means of quantitative estimation or for confirmatory tests provided that prior isolation has been effected by physical and chemical means.

For conclusive identification it is necessary to compare the infra-red spectra of the isolated compound and authentic progesterone. Alternatively, using gas liquid chromatography, the relative retention time of authentic progesterone can be compared to the relative retention time of the unknown steroid.

Chemical methods: These are generally based on the properties described above and consist of extraction of the various tissues with suitable lipid solvents, some

degree of purification and using physical means either the direct measurement of (a) progesterone itself or (b) some chemical derivative of progesterone. The methods utilising the measurement of progesterone itself will be described first.

(a) Measurement of Progesterone Itself: Reynolds and Ginsberg (1942) extracted progesterone by the method of Allen (1932) and then measured progesterone directly by ultra-violet spectrophotometry. The absorption in ultra-violet light depends on the presence of $\Delta 4$ - 3 ketones however, since it was not possible, at that time, to separate progesterone from other $\Delta 4$ - 3 ketones, the method lacked specificity.

Haskins (1950) using a simple ether extraction measured by ultra-violet spectrophotometry the concentration of intravenously injected progesterone in rabbits. The first attempt to isolate progesterone by paper chromatography was made by Haskins, Sherman and Allen (1950) but this was only partially successful.

A further refinement in the purification of progesterone was made possible by the rather elegant paper

chromatographic techniques devised by Zaffaroni (1950) and Bush (1952). Using the aqueous methanol paper chromatographic system of the latter to isolate the progesterone, Edgar (1953[a]) measured progesterone in body fluids and tissues of domestic animals using ultra-violet absorption at 240 mμ. He was unable to detect progesterone in the peripheral blood of the ewe, cow, sow or mare, but measured the relatively high quantities present in the ovarian vein blood of pregnant and cycling ewes and sows (Edgar, 1952, and 1953[b]; Edgar and Ronaldson, 1958).

Independently, Zander and Simmer (1954) using a similar method to that of Edgar (1953[a]) isolated and measured progesterone in various body fluids and tissues of women during pregnancy and during the menstrual cycle. Zander, Forbes, von Münsterman and Neher (1958) using the above method showed that the 20 hydroxy epimers of progesterone had biological activity which partly explained the discrepancy between chemical and bio-assay methods. Zander (1954) identified progesterone in human peripheral blood during pregnancy using infra-red spectrophotometry for final confirmation.

Raeside and Turner (1955) using a combination of

Edgar's and Zander's methods measured progesterone in the ovarian vein blood of the goat and in the peripheral blood of cows injected with exogenous progesterone, but like Edgar (1953[b]) they were unable to detect progesterone in the peripheral blood of cows during the oestrous cycle or during pregnancy.

Progesterone was measured in the corpora lutea of sows and cows in different reproductive states by Loy, McShan and Casida (1957) using chromatography on alumina, counter current distribution and final measurement by ultra-violet absorption. They found a wide range of levels in sow corpora lutea (20 to 105 µg/gm of tissue) but the material was mostly undated. No figures were given for the cow.

The use of sodium hydroxide treatment of plasma prior to extraction with organic solvents was utilised by Short (1958[a]), who employed ether extraction and paper chromatographic techniques as well as direct ultra violet spectrophotometry which resulted in a method of excellent sensitivity, specificity and repeatability. Using this method Short measured progesterone in a wide variety of tissues including plasma in a number of different species. Using very large volumes of plasma he was able to detect

progesterone in the peripheral blood of cattle, sheep, pigs and horses in different reproductive states. In addition he was able to measure plasma progesterone in women throughout the course of pregnancy (Short and Eton, 1959).

Gorski, Erb, Dickson and Butler (1958) using an ethyl acetate : benzene extraction procedure, measured progesterone and its 20β epimer in corpora lutea of cows. They also found progesterone in normal ovarian tissue and in the adrenal glands but were unable to detect it in the placenta of the cow.

Comparatively large amounts of progesterone were isolated from the human placenta using sodium hydroxide treatment prior to ether extraction, followed by column chromatography and counter current distribution (Salhanick, Noall, Zarrow and Samuels, 1952; and Diczfalusy, 1952).

Short (1956) also utilised pretreatment with sodium hydroxide followed by ether extraction, counter current distribution and paper chromatographic separation and studied progesterone levels in the placentae of the domestic animals. He detected progesterone in the placenta of the mare but was unable to isolate if from this source in the cow, sow, ewe

or bitch. Pearlman (1957[a]) and Gorski et al. (1958) were also unable to find progesterone in the placenta of the cow.

(b) Chemical Derivatives of Progesterone: The methods described in section (a) above have all been based on the direct measurement of progesterone by ultra-violet spectrophotometry. Several workers have prepared chemical derivatives of progesterone in an attempt to attain higher sensitivity and specificity in the final reading.

Butt, Morris, Morris and Williams (1951) who extracted with an ethanol:ether (3:1) mixture, purified the extract by column chromatography and measured the Girard derivative of progesterone by polarographic means. By this method they were able to measure accurately the relatively high levels of progesterone in human placental blood. This was the first time that progesterone had been measured in blood with any degree of accuracy.

Pearlman and Cerceo (1952; 1953) using ether extraction after pretreatment with alkali, followed by formation of Girard derivatives, counter current distribution and final estimation of progesterone as the bisthiosemicarbazide derivative were able to measure progesterone in human

plasma as the dinitrophenylhydrazine derivative, but ran into considerable difficulties with contaminating lipid material.

Sommerville and Deshpande (1958), using sodium hydroxide pretreatment of plasma, extracted with ether:methylene chloride (4:1) mixture followed by chromatography on alumina, measured progesterone as the isonicotinic acid hydrazine derivative, which unfortunately has a low molar extinction coefficient and the method therefore lacked sensitivity. (Short, 1961).

Oertel, Weiss and Eik-Ness (1959) using ethanol for extraction and paper chromatography for separation of progesterone, measured the sulphuric acid chromogen of progesterone in ultra violet light. In this way they were able to measure progesterone in the peripheral blood of women during pregnancy and during the menstrual cycle.

Simmer and Simmer (1959) using an extraction and purification method similar to the method described above (Oertel et al. 1959), utilised the thiosemicarbazide derivative for the final estimation of progesterone. They were able to measure successfully the levels of progesterone in

the plasma of women during late pregnancy and the values they found agreed well with those reported in previous studies of this kind.

It will be apparent that many methods have been described for the measurement of progesterone in biological material. Generally speaking most of these methods were very elaborate and, due to emulsion formation, were unsuitable for the processing of large volumes of plasma (Short, 1961). On the other hand the method of Short (1958[a]) was suitable for dealing with the large volumes of plasma necessary for the estimation of circulating progesterone in cows which were the animals to be utilised in this study. (Vide - discussion).

## F. PROGESTERONE IN THE COW.

Progesterone has been demonstrated in many bovine tissues but has not been the subject of many investigations due largely to the difficulty of making accurate measurements of the low levels which occur in this species.

Historical Survey: In the dairy cow the importance of the corpus luteum in the maintenance of pregnancy was demonstrated by Williams (1909) who showed that removal of this structure during pregnancy resulted in abortion. This finding was confirmed by Hess (1920) and Schmaltz (1921).

"Progestins" were identified in large quantities in bovine corpora lutea by bio-assay methods (Kimura, 1935, and Bretschneider, Duyvene and Kaay, 1942).

McNutt (1924) reported the case of an abattoir cow in advanced pregnancy (approximately 230 days) which had both grossly and histologically a small non-functional corpus luteum. This was the first indication that the cow in advanced pregnancy might have an alternative source of "progestins". The same author in a study of the corpus

luteum during pregnancy, McNutt (1927), found that in many cows there was a marked regression during the second half of gestation, with histological changes similar to that seen in the corpus luteum at the end of the oestrous cycle.

A convenient explanation followed when Adler, de Fremery and Tausk (1934) detected progestational activity in extracts of cow placentae using a bio-assay method. This finding was partially confirmed by Ehrhardt and Hardt (1937) who detected "progestins" in one out of three cow placentae.

Uren and Raeside (1951) confirmed experimentally the observation of McNutt (1924) when they showed that the removal of the corpus luteum in a cow 205 days pregnant, and without progesterone replacement therapy, did not interfere with gestation and parturition. In addition it was found that to maintain pregnancy in cows when the corpus luteum was removed prior to 200 days of pregnancy, 100 mg. of progesterone in oil had to be administered daily by intramuscular injection. McDonald, Nichols and McNutt (1952) confirmed these findings and in addition found that in the event of early removal of the corpus luteum replacement therapy could be stopped as early as 140 days without abortion resulting.

It was therefore considered that the placenta of the cow produced progesterone in late pregnancy. However, Short (1956) was unable to detect any progesterone in the placentae of cows during late pregnancy. This was confirmed by Pearlman (1957[a]) and Gorski, Erb, Dickson and Butler (1958). The latter group using ethyl acetate extraction, paper chromatography and ultra violet spectrophotometry, could not detect progesterone in three litres of uterine vein blood from a heifer in late pregnancy, nor in the placentae of six cows. They also confirmed the findings of Gorski, Dominguez, Samuels and Erb (1958) that, in addition to the corpus luteum of the cycle and of pregnancy, both the adrenal gland and the non-luteal ovarian tissue contained progesterone. In addition they detected 20B hydroxy progesterone, a metabolite of progesterone, in luteal tissue from pregnant and non-pregnant cows. This conversion has been shown to occur <u>in vitro</u> in cow luteal tissue homogenates with added progesterone (Hayano, Lindberg, Weiner, Rosenkrantz and Dorfman, 1954).

Englehart as early as 1930, noticed progestational activity in young rabbits injected with lipid extracts of the adrenal gland. Callow and Parkes (1936) prepared extracts from the adrenals of most of the domestic animals which

produced full progestational response in rabbits. The chemical identification of progesterone from the ox adrenal was reported by Beall and Reichstein (1938).

Balfour, Comline and Short (1957) isolated progesterone from the adrenal vein blood of pregnant and non-pregnant cows, calves and steers. Short (1960[a]) estimated that the adrenal glands of the pregnant cow could secrete as much as 1.5 mg/hour, which would represent a significant contribution to the maintenance of pregnancy. However, he pointed out that a proportion of this might result from surgical stress during canulation of the adrenal vein.

Balfour, Comline and Short (1959) have shown that the adrenal gland of the newborn calf secretes only 20 $\alpha$ hydroxy progesterone but that there is a gradual change to progesterone itself which is complete by about 2 months of age. The significance of this is at present unknown but suggests that an enzymic factor is involved.

Short (1957) made a few preliminary observations on plasma progesterone levels of the cow during the oestrous cycle. He detected the hormone in two out of five samples examined.

Short (1958[b]) using large volumes of plasma measured progesterone in the peripheral blood of cattle throughout the course of pregnancy and found that the level was of the order of 1 µg/100 ml until three weeks prior to parturition. At this time it began to fall and was undetectable at calving.

Mares and Casida (1960) measured by the chemical method of Loy et al. (1957), the levels of progesterone and the 20β epimer in the corpus luteum during the oestrus cycle in the heifer. They based their finding on the estimation of corpora lutea obtained per vaginum from two heifers on days, 7, 9, 11, 13, 15 and 17 of the oestrus cycle. Progesterone concentration was found to increase from 26 µg/gm on day 7 to a maximum of 45 µg/gm on day 15, when 20β hydroxy progesterone was also at its highest. There was little difference in corpus luteum weights on the various days of the cycle.

Zimbelman, Loy and Casida (1961) studied the progesterone level in cow corpora lutea on days 14, 18, 23, 28 and 42 of pregnancy. They found the highest levels on day 14 (35 µg/gm or 200 µg/gland) with a gradual fall in level with advancing pregnancy. The quantity of 20β hydroxy

progesterone did not vary with the stage of pregnancy.

Several groups of workers have reported the levels of progesterone in the corpus luteum throughout the course of pregnancy. There appears to be some divergence of results, both in the levels found in the glands, and the stage of pregnancy at which the maximum level was attained. Kaay (1942) using a bio-assay method found that the maximum level was reached during the 3rd month of gestation and this was subsequently confirmed by a chemical method of estimation (Stormshak and Erb, 1961). Melampy, Hearn and Rakes (1959) and Kristoffersen (1960), both using chemical methods found maximum levels during the fourth month and fifth month of pregnancy, respectively. The concentration of progesterone varied with the method used but generally fell within the range of 10 to 25 µg/gm of tissue.

In a similar study to that of Mares et al. (1960), Mares, Zimbelman and Casida (1962) studied the levels of progesterone in the corpus luteum during the oestrus cycle in the cow on days 3, 5, 7, 9, 11, 13, 15, 17 and 19 days. They reported low levels of progesterone and the 20β epimer on days 3, 5 and 7 when on day 9 there appeared to be a

marked increase from 25 µg to 35 µg per gram of luteal tissue. The peak level of 45 µg/gm was reached on day 15 of the cycle and fell sharply on day 17 to 7 µg/gm although there was not such a marked reduction in corpus luteum weight at this time.

Loy, Zimbelman and Casida (1960) and Zimbelman, Loy and Casida (1961) have studied the effects of administered ovarian hormones in corpus luteum function and development in the cow. The general effect was that the progesterone concentration in the corpus luteum was depressed when exogenous progesterone was given but the weight of the gland remained unchanged. When oestrogens were administered the corpus luteum regressed prematurely.

The relative abundance of progesterone compared to other steroids in follicular fluid of the cow was reported by Short (1962) in contrast to his previous findings in equine or human follicular fluid (Short, 1960; Short and London, 1961). However, the author pointed out that this high level of progesterone could be partly due to contamination with fluid from luteal cysts.

Early embryonic death in cattle has been reported

as a major cause of infertility by a number of different workers. (Tanabe and Casida, 1949; Laing, 1949; Hawk, Wiltbank, Kidder and Casida, 1955; Asdell, 1958 and Greenstein and Foley, 1958). In normal cows the general finding was that a few days after service fertilised ova were present in approximately 90%, while after one month, the percentage of cows pregnant had fallen to approximately 60%. In cows known to have diminished fertility (repeat-breeders) the figures were 60% and 30% at 3 days and one month respectively. This constitutes a major production loss to the agricultural industry.

In view of the fact that progesterone is so intimately concerned with embryonic life a number of trials have been run to observe the effect of exogenous progesterone on conception rates and embryonic mortality in selected groups of cattle. Various workers have claimed different degrees of success with progesterone therapy for improving conception rates in "repeat-breeders" and normal cows (Herrick, 1953; Dawson, 1954; Wiltbank, Hawk, Kidder, Black, Ulberg and Casida, 1956; Johnson, 1958 and Hansel, McEntee and Wagner, 1960).

Some preliminary studies have been carried out

on the relationship between embryonic death and the progesterone content of the corpora lutea in normal and "repeat-breeder" cows.

Using the method of Stormshak, Hunt and Erb (1961) which incorporates the use of an internal marker of progesterone - 4 - C14, Erb and Stormshak (1961) estimated the levels of progesterone and 20β hydroxy progesterone in the corpus luteum, ovaries and adrenals in the normal and repeat-breeder cow during the cycle and during early pregnancy, and found the levels to be essentially the same. The total quantity of progesterone was less than 100 µg until after the sixth day of oestrus. Maximum quantities were observed from days 14 to 16 of the cycle with six cows averaging 251 µg per gland. Total progesterone did not decline until near the time of expected oestrus. During pregnancy the average total gland level for seven cows at 25 to 34 days pregnant was 161 µg while at 37 to 42 days it was 250 µg.

Foote, Zimbelman, Loy and Casida (1959) using the progesterone estimation method of Loy et al. (1957) studied the progesterone levels in corpora lutea removed per vaginum from first service and repeat-breeder heifers on day 14 of the cycle. Results indicated that there was no significant

difference between the two groups of seven animals used, in the gland weight, progesterone content and proportion of functional luteal cells. The study was repeated on each animal following the elapse of one normal cycle but there was again no difference between the two groups and no difference from the previous results except that the weight of the first corpora lutea was somewhat heavier on average than the second. The progesterone concentration ranged from 18 to 80 µg/gm and the total amount per gland from 80 to 354 µg.

Staples and Hansel (1961) studied the progesterone levels in the corpora lutea of heifers in relation to embryo survival at 15 days after mating. From a group of 27 animals the conception rate was 74% and there was no significant difference between the pregnant and non-pregnant animals in the levels of progesterone present in the corpora lutea. However the levels of 20β hydroxy progesterone appeared to be substantially higher in the non-pregnant heifers. These workers also studied the effects of daily oxytocin injections in a similar group of heifers and found a much reduced conception rate (40%) with most animals showing signs of precocious ovulation

prior to slaughter on the 15th day. In those animals which were pregnant the progesterone content of their corpora lutea did not differ from the levels found in the untreated group.

## G. PROJECT AND PLAN OF EXPERIMENTS.

The object of this study was firstly to establish normal blood progesterone levels for the cow during the oestrous cycle. In addition it was hoped to establish tissue levels and study the metabolism of progesterone in the cow at this time.

Following the preliminary study the intention was to examine the distribution of progesterone in inseminated cows in an attempt to establish whether this hormone was related to conception rates or possible embryonic mortality.

It was fortunate at this time that a study of embryonic death in cattle was due to commence in the University of Glasgow Veterinary Hospital. This project was financed by the Agricultural Research Council and was under the direction of Drs. Boyd, Baosich and Young. The object of this study was to compare the histology of the endocrine glands and reproductive organs of cows, pregnant and non-pregnant at 16 and 26 days after insemination. In addition immunological and bacteriological aspects were to be studied.

During the period of study it was hoped to examine, at the Veterinary Hospital, clinical cases which might be influenced by progesterone or its metabolites.

The methods selected for the determination of progesterone in plasma and in corpora lutea were those of Short (1958[a]) and Rowlands and Short (1959) respectively. For the estimation of progesterone in body fat, the extraction and purification procedures were based on those of Allen (1932).

Owing to the difficulty of estimating progesterone in bovine tissues a considerable amount of time was to be devoted to the examination of the methods employed. This was particularly necessary with bovine plasma where, as a result of gel formation, difficulties can be encountered during the extraction procedure. In addition polar lipid impurities on the chromatograms can create problems which are not normally encountered in other species. (Short, 1958[b]; 1960, personal communication).

## SECTION 2 - METHODS

A. REAGENTS

B. APPARATUS

C. MATERIALS

D. PROCEDURES

## SECTION 2 - METHODS

**Introduction:** The methods were based essentially on those of Short (1958[a]) and Rowlands and Short (1959) with some modifications, notably the incorporation of an internal radioactive marker of radio-progesterone (progesterone - 4 - C14). With bovine plasma samples it was found necessary to acetylate, then re-chromatograph the progesterone spot.

### A. REAGENTS.

**Peroxide Free Ether:** This was prepared by the method of Brown (1955) in which diethyl ether B.P. Solvent was washed with a twentieth volume of a saturated solution of ferrous sulphate followed by three 1/10th volume washings with distilled water. Final purification was carried out by distillation at 38°. The peroxide free ether was stored in dark bottles and was used within a fortnight.

**Ethyl alcohol:** was "Special for Spectroscopy" (James Burrough Limited, London). This was not re-distilled before use. All other organic solvents were re-distilled before use on a 60 centimetre fractionating column packed with single turn glass helices.

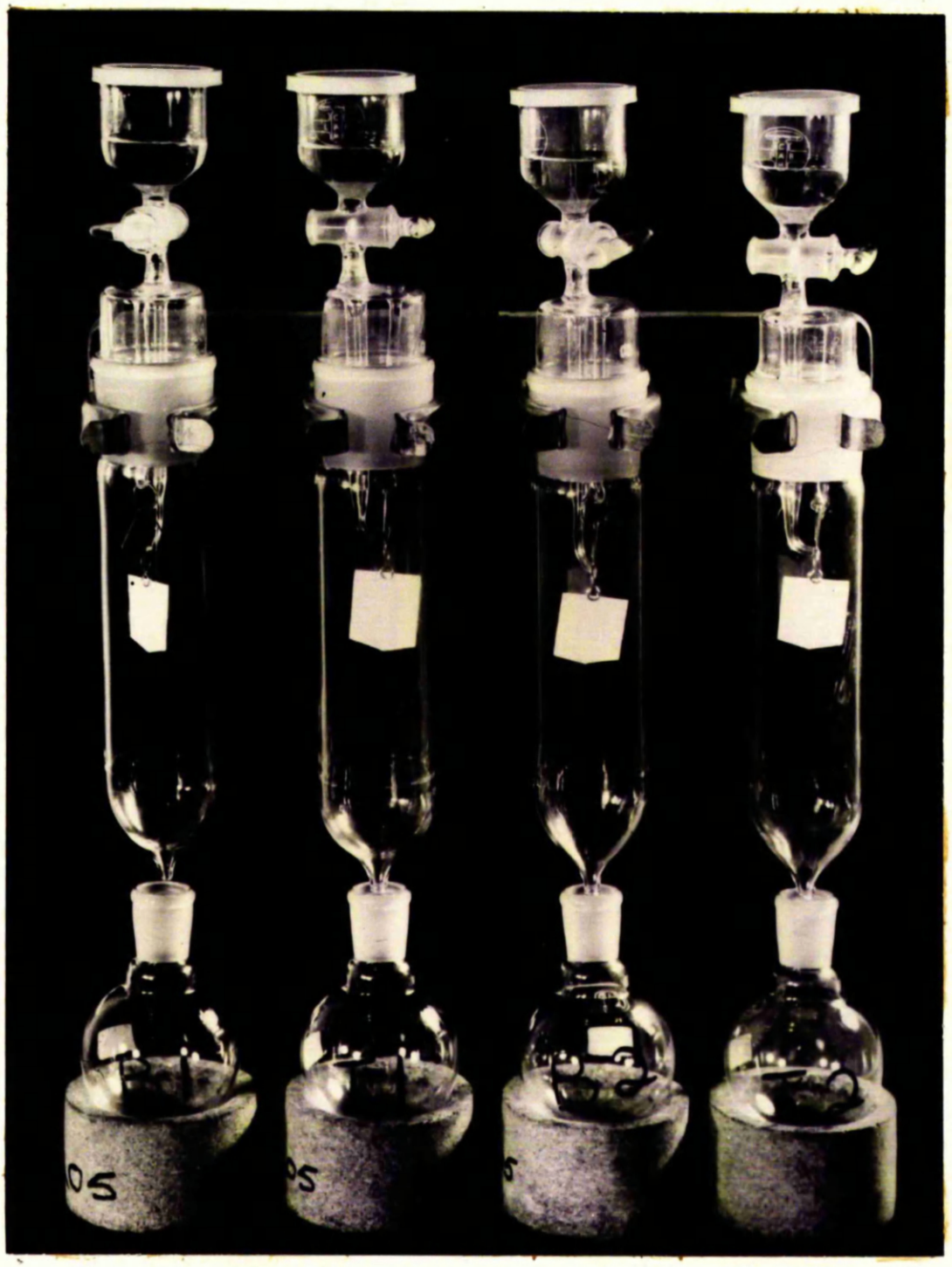
Radio-Active Progesterone (progesterone - 4 - C14) was obtained from the Radio Chemical Centre, Amersham. The specific activity was 27.9 μc/milligram with a radio chemical purity of 102.0%.

Marker Steroids were obtained from the M.R.C. Steroid Reference Collection, through the courtesy of Professor W. Klyne.

All other chemicals used were of Analar grade.

FIGURE 2.B.1.

ELUTION APPARATUS.



## B. APPARATUS

Glass Homogenisers were made by Wesley Coe Co. Ltd., Cambridge. They were 10" in length and made to the specification of Dr. L.T. Samuels.

A rotary film evaporator (Quickfit and Quartz) was used to evaporate methanolic extracts to dryness.

Chromatography tanks were obtained from Shandon Scientific Co. Ltd., London. The type used was the Universal 13" Strip Chromatanks.

A Hanovia "Chromatolite" Ultraviolet Lamp emitting at 2537A was used in ultra violet contact photography.

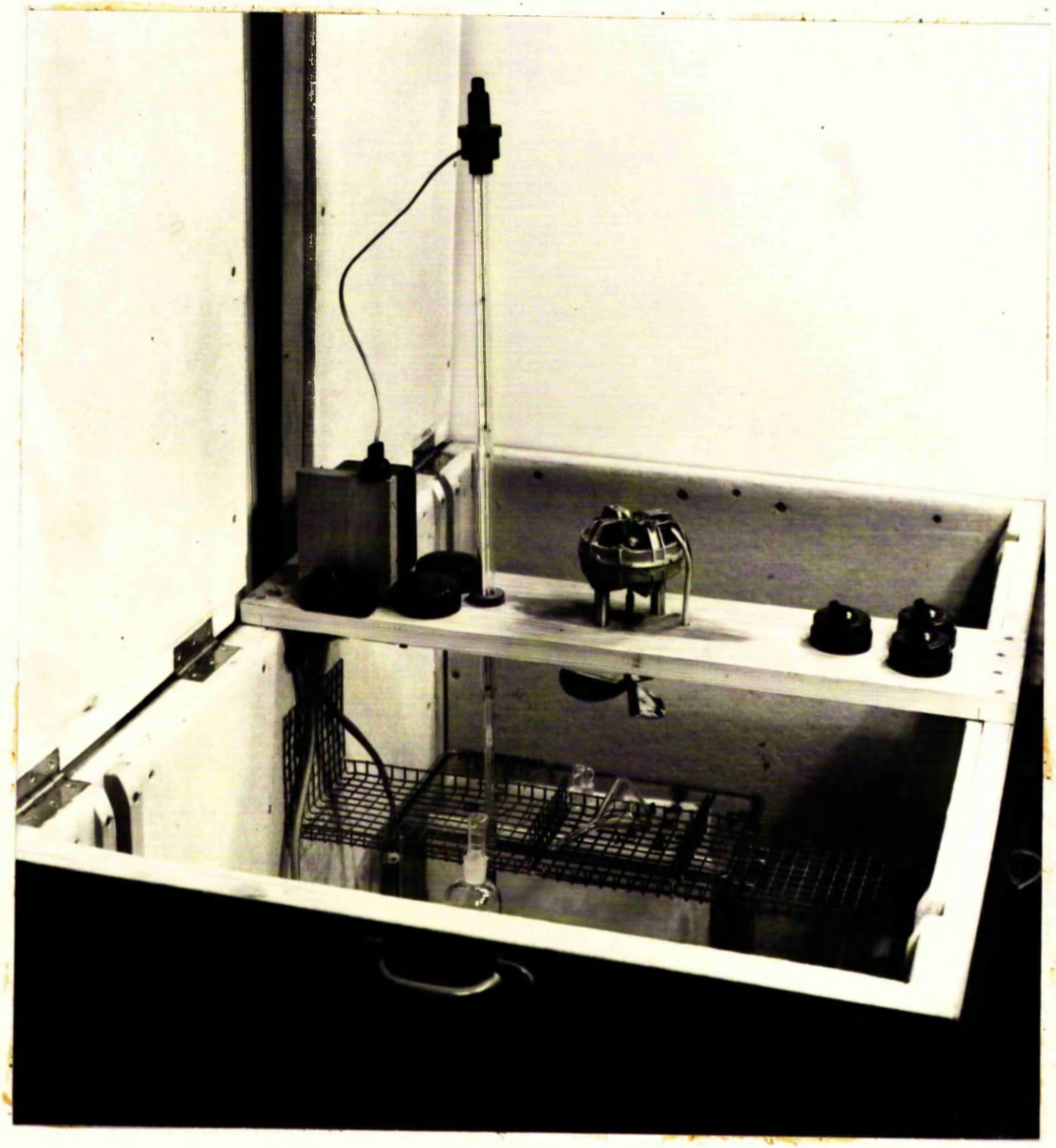
Elution Apparatus was a modified version of that described by Zander and Simmer (1954), made by Camlab Ltd., Cambridge. See Figure 2.B.1.

A Unicam S.P.500 spectrophotometer was used for final reading of samples.

Quarts microcells of capacity of 0.4 ml and an optical path of 1 cm. were used in conjunction with a quartz focusing lens.

FIGURE 2.B.2.

CHROMATOGRAPHY CABINET



Carbon 14 counting equipment Carbon 14 was measured with a thin mica end-window G.M. tube type E.W.3H. (20th Century Electronics) and a Panax Scaler type D. 657.

Chromatography Cabinet This was used for temperature control of chromatography tanks and was made by Mr. Guntrip, Senior Technician in Animal Research Station, Huntingdon Road, Cambridge, through the courtesy of Dr. T.R.R. Mann. Vide photograph - Figure 2.B.2.

Glassware Most of the glassware was supplied by Quickfit and Quartz Ltd. This included distillation apparatus, separating funnels, condensers, equipment fitted with ground glass joints.

CLEANSING OF GLASSWARE:

All glassware used in the determination was soaked overnight in chromic acid (Conc. $H_2SO_4$ : Saturated potassium dichromate - 1:1). Several washings in warm water was followed by a two hour immersion in an 0.2% solution of sodium sulphite - acidified with sulphuric acid to remove any remaining traces of chromic acid. (Brown J.B., 1955). After repeated washings with warm water and a final rinse with de-ionized water the apparatus was dried in a hot air oven at 110°.

## C. EXPERIMENTAL MATERIALS.

The tissues used in the present study were bovine plasma and bovine corpora lutea. A small number of bovine body fat and milk samples were also utilised. The blood samples were collected from the jugular vein by means of a canula with sodium oxalate (0.25%) as an anticoagulant. The blood was spun down immediately in an M.S.E. medium refrigerated centrifuge at $0^{o}$C for 1 hour at 2,000 x G. Blood samples which were obtained at a distance from the hospital were collected over ice and stored at $0^{o}$C until the plasma could be separated. Plasma samples were stored in a deep freeze at $-15^{o}$C.

The majority of the corpora lutea were obtained at slaughter. These were carefully dissected from the ovary, weighed, and a portion weighing approximately 0.5 gm was removed for assay. A small number of corpora lutea were removed from the live animal per vaginum under posterior epidural anaesthesia. The animal was restrained in a "crush" and 4 cc of a 5% solution of procaine hydrochloride injected at the sacro-coccygeal junction. Anaesthesia followed within minutes and involved the buttock, perineum, vulva, vagina and tail. The vaginal floor was

depressed by means of a long metal probe and an incision of 3.5 cms. was made with a guarded blade in the dorsal anterior vaginal fornix. The depression of the vaginal floor was a precaution against accidental damage to the aorta or rectum. By rectal manipulation the ovary, containing the corpus luteum, was guided through the incision into the vagina where the corpus luteum was enucleated. The ovary in this situation could be inspected by means of a vaginal speculum.

Bullock blood was collected over ice at Glasgow Abattoir and conveyed to the laboratory where separation of the plasma was carried out at $0^{o}C$.

## D. PROCEDURES

The procedures can be considered in the following steps:

1. EXTRACTION OF TISSUE: crude lipid extraction of plasma, corpus luteum or other tissues.

2. PAPER CHROMATOGRAPHY: separation of steroids from other lipids.

3. ULTRAVIOLET CONTACT PHOTOGRAPHY: location of steroid "spots" on chromatogram.

4. ELUTION: recovery of steroid from paper using elution apparatus described above.

5. SPECTROPHOTOMETRY: quantitative estimation of steroid in Unicam S.P. 500 at 220, 240 and 260 millimicrons.

6. CARBON $^{14}C$ COUNTING: measurement of recovered radio activity direct from test solution in quartz microcells.

## FLOW SHEET FOR EXTRACTION OF PLASMA.

BLOOD PLASMA

Treat with ½% NaOH add 1000 c.p.m. progesterone - 4 - C14 and extract 2, 3 or 4 times with an equal volume of ether.

→ PLASMA RESIDUE

ETHER EXTRACT

Wash with 1/10 volume distilled water to remove traces of NaOH.

→ WATER SOLUBLE RESIDUE.

WASHED ETHER EXTRACT

Evaporate to dryness, redissolve in light petroleum and extract with 70% aqueous methanol.

→ LIGHT PETROLEUM RESIDUE.

METHANOLIC EXTRACT

Evaporate to dryness on rotary film evaporator and transfer to 2 ml glass tube with washings of absolute methanol.

CHROMATOGRAPHY

This is followed by elution, acetylation, re-chromatography and measurement in spectro-photometer. Radioactivity in sample counted and recovery % calculated.

1. EXTRACTION OF TISSUE:

(A) PLASMA: 500 ml bovine plasma was treated with $5^{N}$ sodium hydroxide to give a final concentration of ½%. 0.18 µg of progesterone - 4 - C14 (approximately 1000 counts per minute) was added in 0.2 ml benzene by means of an auto zero pipette. A standard planchet containing the same amount of radio-progesterone was prepared at the same time.

The plasma sample was stirred with a glass rod to ensure thorough mixing of radio-active progesterone, plasma and added sodium hydroxide and then extracted 2, 3, or 4 times with an equal volume of peroxide free ether. Ether extracts were pooled, washed with a 1/10th volume of distilled water to remove traces of sodium hydroxide, and concentrated to near dryness in a waterbath at $70^{0}$. The last traces of ether were removed in a stream of nitrogen over a warm water bath.

During the ether extraction of the plasma from certain individual cows there was a tendency for the formation of a gel which varied from a slight thickening or increased viscosity to a solid table-jelly like formation.

## FLOW SHEET FOR EXTRACTION OF LUTEAL TISSUE.

**LUTEAL TISSUE**

Homogenise luteal tissue with 4 x 5 ml portions of 2½% NaOH W/V. Add 1000 c.p.m. of progesterone - 4 - C14 and extract homogenate with 6 x 14 volumes of ether.

→ TISSUE RESIDUE

**ETHER EXTRACT**

Wash with 1/10 volumes of water until neutral to litmus.

→ WATER SOLUBLE RESIDUE

**WASHED ETHER EXTRACT**

Evaporate to dryness, redissolve in light petroleum and extract with 70% aqueous methanol.

→ LIGHT PETROLEUM RESIDUE

**METHANOLIC EXTRACT**

Evaporate to dryness on rotary film evaporator and transfer to 2 ml. test tube with washings of absolute methanol.

**CHROMATOGRAPHY**

When gel formation occurred it was usually after the first ether extraction had been taken and it was noticed in both fresh and deep frozen samples. It occurred more commonly in the first two or three samples taken from individual cows then it seemed to taper off following a slight increase in the plasma yield from these cows. It also occurred in one cow (which had not exhibited this phenomena previously) following a course of injections with chorionic gonadotrophin. (L.H. Mainly).

The difficulty of the gel formation was overcome by adding 100 ml of distilled water to the "gel" plasma and gently stirring until the plasma had re-dissolved whereupon the extraction process continued with extra ether to make up for the increase in volume.

B. CORPORA LUTEA: The extraction method used was based on that of Rowlands & Short (1959). The luteal tissue was homogenised in a glass homogeniser with four 5 ml. volumes of 2½% NaOH (W/V). 0.18 µg radio-progesterone (1000 c.p.m.) contained in 0.2 ml benzene was added to the pooled homogenate which was extracted six times with 25 ml ether. A standard planchet containing the same amount of radio progesterone was prepared at the same time. The

## FLOW SHEET FOR THE EXTRACTION OF BODY FAT.

BODY FAT

To each 100 gms of homogenised fat add 10 ml 5N NaOH, mix and allow to stand for 30 minutes. Extract 3 times with an equal volume of ether.

→ FAT RESIDUE

ETHER EXTRACT

Wash pooled ether extract with 1/10 volume distilled water to remove traces of NaOH and evaporate to dryness.

→ WATER SOLUBLE RESIDUE

WASHED ETHER EXTRACT

Extract residue twice with 400 ml 70% methanol at 25°C freeze to -15° and filter cold.

→ ETHER SOLUBLE FAT RESIDUE

METHANOLIC EXTRACT

Evaporate to dryness and transfer with 3 washings of absolute methanol to a 5 ml centrifuge tube. Redissolve in 5 ml. warm 70% methanol and freeze at -15° overnight. Centrifuge at 2,000 G in the morning and decant supernatant which is evaporated to dryness.

CHROMATOGRAPHY

In most cases acetylation and rechromatography was necessary to purify the sample.

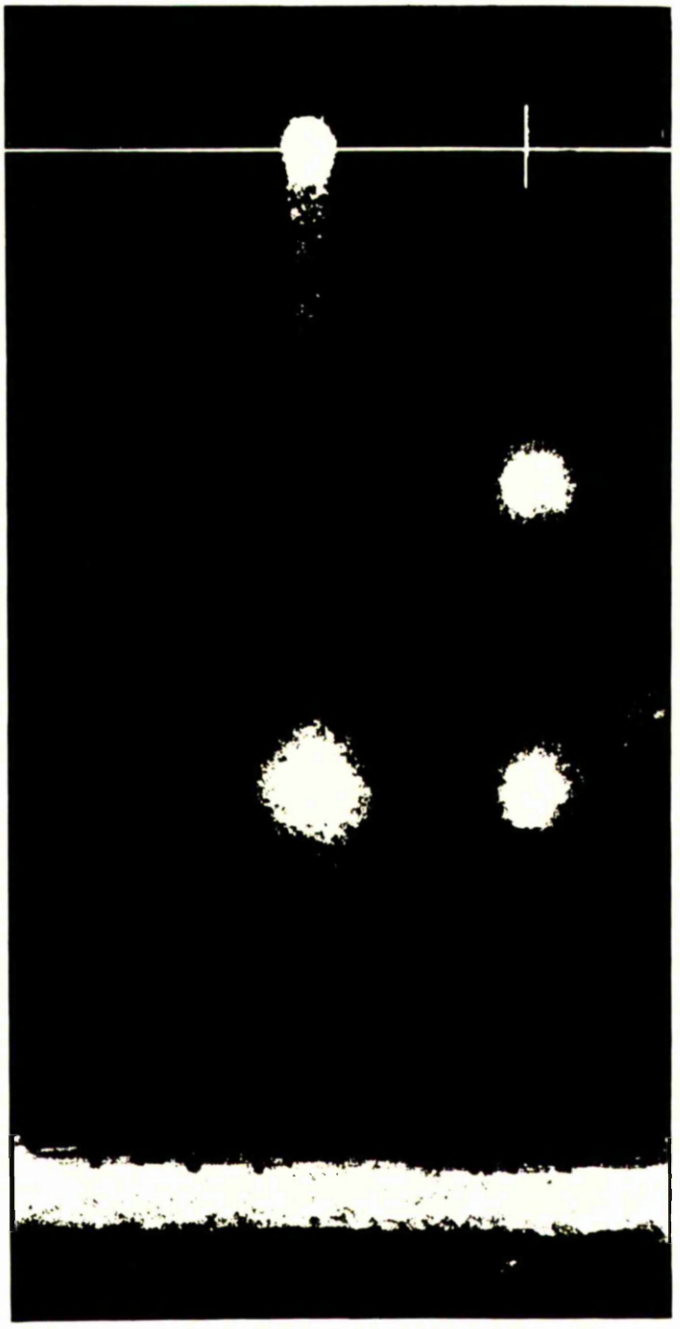
ether extracts were pooled and washed three times with 2 ml portions of distilled water or until neutral to litmus paper. The ether extract was then evaporated to partial dryness and final traces of ether were removed with a stream of nitrogen in a warm water bath at 50°.

The dried ether extracts of either plasma or corpora lutea were then treated in the same way. They were taken up in 3 x 10 ml volumes of light petroleum (40° - 60°) and transferred to a separating funnel. The light petroleum containing the lipid extract was then extracted six times with 10 ml 70% methanol (Allen, 1932). This allowed for separation of the steroids from the pigments and crude lipids contained in the light petroleum. Progesterone under this system has a partition co-efficient of one (1) so that theoretically the methanolic extract contains 98.4% of the available progesterone.

The methanolic extract contained in a 500 ml round bottom flask was then taken to dryness on a rotary film evaporator using a water bath at 60°C and a reduced pressure of approximately 60 mm of mercury.

The residue was dissolved in 1 ml absolute methanol, transferred to a 3 ml glass test tube, and evaporated to

FIGURE 2.D.1.




PERIPHERAL BLOOD COW MID CYCLE

720 ML PLASMA.

dryness under a stream of nitrogen in a water bath at 50°. This was repeated using one further washing of 1 ml methanol and lastly one of 0.5 ml methanol.

C. BODY FAT: Using pre-treatment with sodium hydroxide and the freeze-precipitation methods employed by Allen (1932), body fat from cows was examined for the presence of progesterone. The initial purification steps are shown in the flow sheet for body fat: the final acetylation and chromatography being identical to that used for other tissues.

2. CHROMATOGRAPHY: Preparation of paper: Whatman chromatography paper No.20 in strips measuring 11.5 x 42.5 cms. were washed by descending flow in absolute methanol for 48 hours and then allowed to dry in air. The residue in the test tube was dissolved in 0.05 ml absolute methanol and applied to the paper in strips using a hot air blower. In order to ensure the complete transfer of the residue to paper, two further washings of 0.025 ml absolute methanol each were used and applied to the paper as before. Marker steroids are shown in Figure 2.D.1.

The paper was placed in the chromatography tank in such a way that the origin line was placed 6 cms. from

the trough edge and the first 4 cms. of the paper was horizontal (Bush, 1952). The paper was equilibrated in the tank for 1 hour at 25° before the solvent mobile phase was run in. The solvent system used was Bush type A, 80% methanol : light petroleum (80° - 100°C) 1:1 (Bush, 1952). The additional system of -70% Methanol : Hexane (60° - 80°) was found to give a good separation of progesterone from the solvent front when this was required.

When a new tank was set up for chromatography about 200 ml. of each solvent was placed in separate containers in the tank and an equilibration period of 18 hours was allowed before any chromatography was carried out in this particular tank. (Clayton, 1956).

The chromatograms were normally developed with 100 ml of mobile phase and a descending flow of solvent. The time allowed for development was 2½ hours and the papers were dried at room temperature in air.

3. ULTRAVIOLET CONTACT PHOTOGRAPHY: Dry chromatograms were placed on top of a sheet of contact paper (Kodak Duostat Reflex 13) and pinned to a thin wooden board which was then flexed to ensure good contact of the papers during

exposure to ultraviolet light. Photography was carried out at a distance of 20 cms. from the source (Hanovia Chromatolite) for a period of 3 seconds. The contact photographs were developed, fixed, and washed in the usual manner then dried in a curved print drier to prevent shrinkage or distortion. Steroids possessing a $\Delta 4 - 3$ Ketone ($\alpha\beta$ unsaturated Ketone) group showed up as white spots on a dark background. Figure 2.D.1. (Fisher, Parsons & Morrison, 1948; Markham & Smith, 1949; Bush, 1952; and Haines, 1952). The whole procedure was carried out in a photographic dark room with normal safety conditions of lighting.

4. ELUTION: The chromatogram was re-applied to the dry print and correct apposition was achieved by the pin holes present. The exact location of the steroid was determined by holding the two papers against an ordinary light source. A faint pencil line was drawn round the spot which was then cut out. A paper blank of the same dimensions was taken from an area the same distance from the origin as the test spot, i.e. of the same Rf. value. The bottom edge of the areas removed were cut to form a blunt point as described by Bush (1961), then suspended by means of stainless steel

clips in the elution apparatus described above. 5 ml absolute methanol was then allowed to drip slowly onto the papers and the flow was adjusted so that elution was complete in 20 minutes. The eluates were collected in 100 ml round bottom flasks.

5. SPECTROPHOTOMETRY: The test and blank eluates were evaporated to dryness under reduced pressure in a water bath at 50°C. The residues were re-dissolved in 1 ml ethanol and transferred to the quartz microcells. Absorption measurements were made at 220, 240, and 260 millimicrons in a Unicam S.P. 500 spectrophotometer. The formula of Allen (1950) was used to compensate for linear background absorption due to impurities. The corrected absorption can be expressed as follows:

$$\text{Corrected optical density at 240 m}\mu = \text{O.D.240} - \frac{(\text{O.D.220} + \text{O.D.260})}{2}$$

The amount of progesterone present in the test solution was calculated from the standard calibration curve shown in Figure 3.A.2.

6. CARBON 14 COUNTING: Immediately following spectrophotometry 0.2 ml of the test solution was removed from the

microcell by means of an auto-zero pipette, run into a nickel planchet, and allowed to dry with gentle heating. Carbon 14 activity on the test planchet was determined in the counting equipment described above. The standard planchet, containing the amount originally added, was counted before and after the test planchet. From these findings the percentage recovery of the radioactive tracer was calculated and this was taken to be the same as the recovery of any progesterone present in the original sample.

The precautions observed in the use of $C^{14}$ were those described in The Code of Practice of Persons Exposed to Ionising Radiation in University Laboratories (1961).

## SECTION 3 - RESULTS AND DISCUSSION

A. EXAMINATION OF METHODS.

B. PROGESTERONE IN THE CYCLING COW.

C. PROGESTERONE LEVELS IN THE COW DURING EARLY PREGNANCY.

D. PROGESTERONE LEVELS IN COWS WITH CERTAIN CLINICAL CONDITIONS.

## SECTION 3 - RESULTS & DISCUSSION

### A. EXAMINATION OF METHODS.

In this section the results obtained during the preliminary study of the method are presented under the following headings:

(1) Absorption spectrum of progesterone and the use of the Allen Correction.

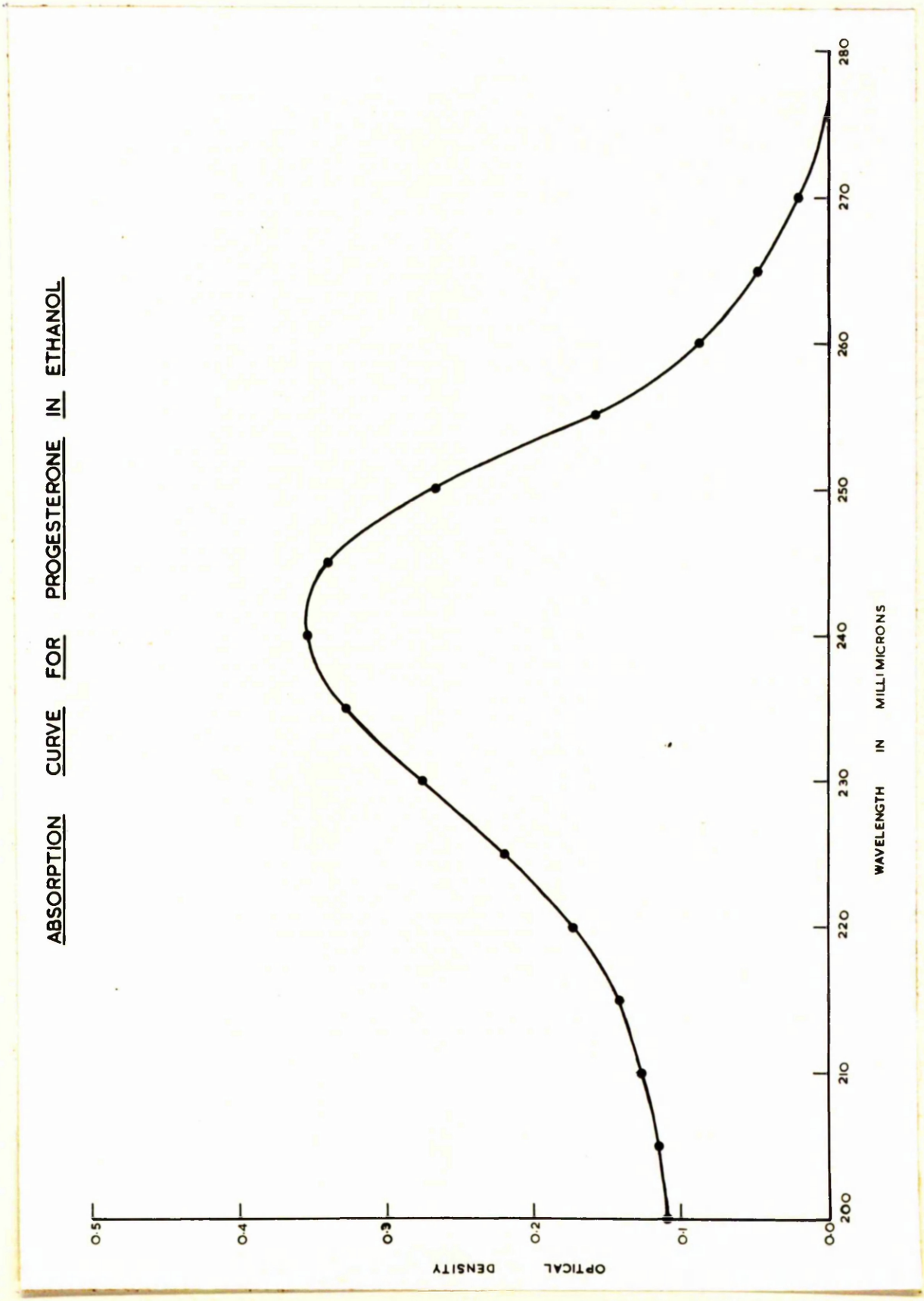
(2) Preparation of concentrated solution and construction of calibration curve.

(3) Recovery of progesterone from bovine plasma.

(4) Recovery of progesterone added at various steps in the procedure.

(5) The use of progesterone - 4 - C14 as an internal marker.

FIGURE 3.A.1.


ABSORPTION CURVE FOR PROGESTERONE IN ETHANOL
OPTICAL DENSITY
0·0
0·1
0·2
0·3
0·4
0·5
200
210
220
230
240
250
260
270
280
WAVELENGTH IN MILLI MICRONS

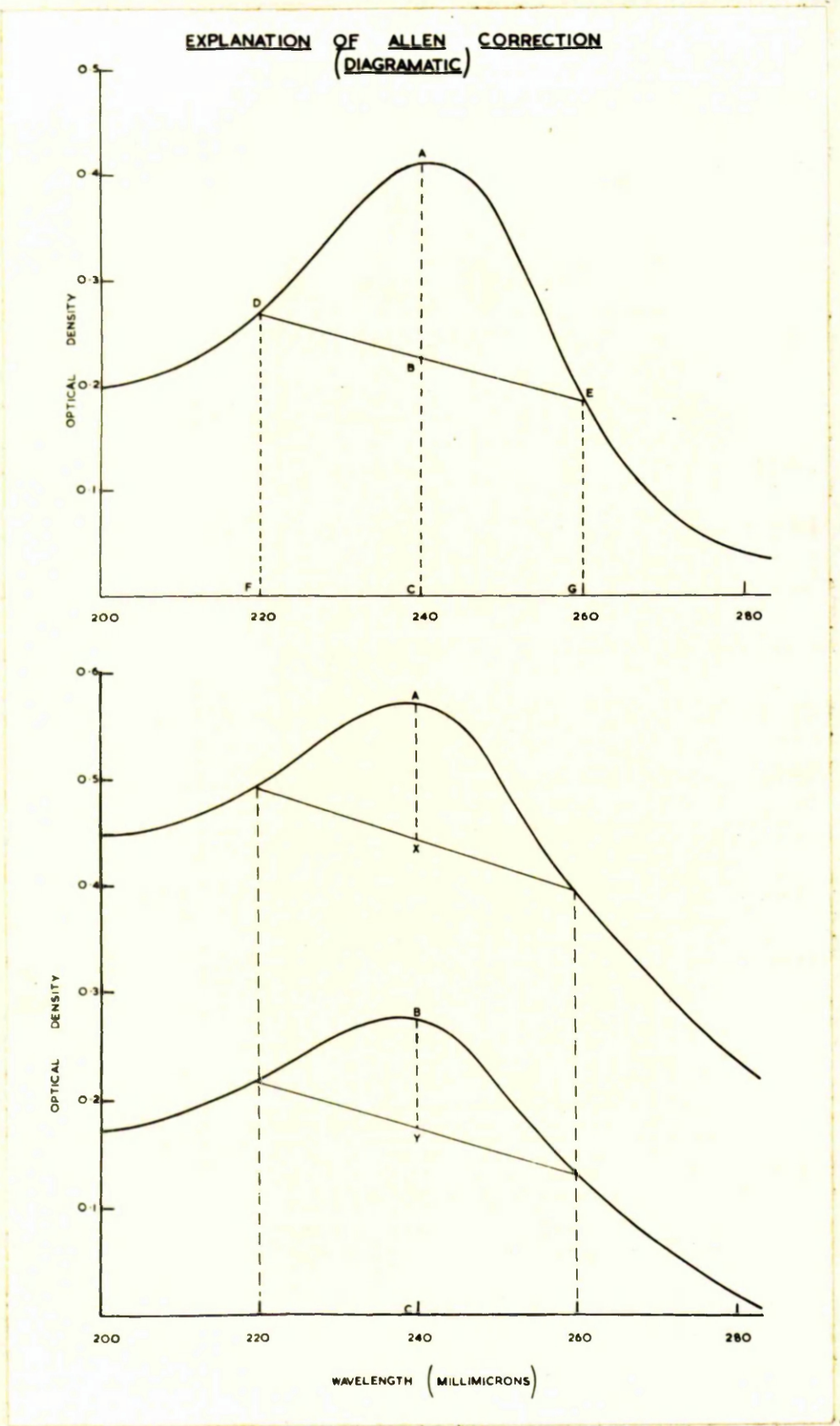
## SECTION 3 - RESULTS AND DISCUSSION.

### A. Preliminary Study of Methods.

#### (1) Absorption spectrum of progesterone in ethanol including notes and diagrams on the use of the Allen Correction:

A compound absorbs light of different wave lengths to a varying degree depending on the chemical nature of the compound under investigation, e.g. there is intense absorption of light at 240 millimicrons (mμ) in the case of steroid Δ4 - 3 ketones including progesterone. An absorption spectrum can thus be determined by plotting optical density against wave length. The absorption spectrum of progesterone in ethanol is shown in Figure 3.A.1. It should be noted here that an identical curve is produced by many other steroids possessing the Δ4 - 3 ketone grouping. Thus without prior chemical isolation, in this case by paper chromatographic means, U.V. absorption is non-specific. It is also worth recalling here that this property of U.V. absorption is used for detection of progesterone and its 20 hydroxy epimers on paper chromatograms as described above on page 58 under methods.

# FIGURE 3.A.2.


EXPLANATION OF ALLEN CORRECTION
(DIAGRAMATIC)
a.
OPTICAL DENSITY
0·5
0·4
0·3
0·2
0·1
A
D
B
E
F
C
G
I
200
220
240
260
280
b.
OPTICAL DENSITY
0·6
0·5
0·4
0·3
0·2
0·1
A
X
B
Y
C
I
200
220
240
260
280
WAVELENGTH (MILLIMICRONS)

In order to obtain maximum sensitivity during spectrophotometric estimation it is essential to use the wave length at which maximum absorption occurs, in this case 240 millimicrons. This is termed $\lambda_{\frac{max.}{240}}$.

Should, however, there be present other material which absorbs light in the $\lambda$max. region then it is necessary to correct the optical density reading for this interference. The method used to correct for these impurities is that described by Allen (1950) and is based on the provision that the absorption by the impurities is of a linear nature over the required wave length range. Thus, by measuring the optical density at wave length equidistant from the absorption maximum, and subtracting the average of the two from max. a new figure is obtained which is more representative of the optical density of pure progesterone at this concentration. This is shown diagramatically in Figure 3.A.2[a].

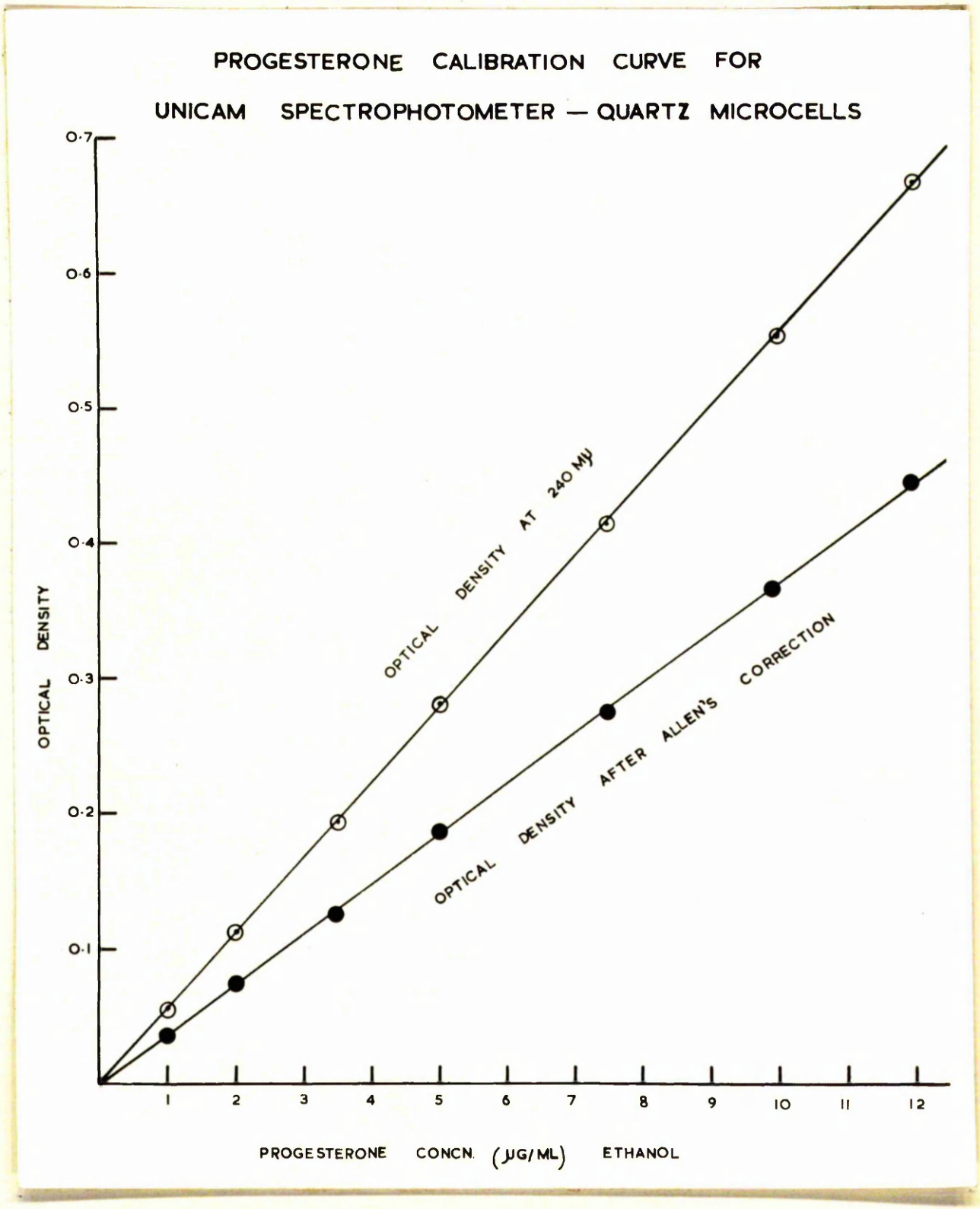
The Allen Correction can be represented by the formula:

$$\text{Corrected Optical Density} = AC - \frac{(DF + EG)}{2} = AC - BC = AB$$

To demonstrate the usefulness of this procedure a hypothetical case of two absorption curves of progesterone

is shown in Figure 3.A.2.[b] At first sight the upper curve would appear to represent a higher concentration of progesterone than the lower one because AC is much greater that BC. However, after the application of the Allen Correction to each curve it will be seen that the optical densities of the two solutions are represented by the lines AX and BY. Therefore the corrected calibration curve is based on the apparent peak (AX or BY) and not so much on the total height of the curve (AC or BC).

FIGURE 3.A.3.


PROGESTERONE CALIBRATION CURVE FOR
UNICAM SPECTROPHOTOMETER — QUARTZ MICROCELLS
OPTICAL DENSITY
0·7
0·6
0·5
0·4
0·3
0·2
0·1
OPTICAL DENSITY AT 240 MU
OPTICAL DENSITY AFTER ALLEN'S CORRECTION
1
2
3
4
5
6
7
8
9
10
11
12
PROGESTERONE CONCN. (UG/ML) ETHANOL

(2) Preparation of concentrated solution and calibration curve.

Progesterone was obtained from Messrs. Organon Ltd., and this was checked for chemical purity by chromatographing approximately 100 µg using Bush System A. It was established that there was less than 1% contamination with either of the 20 hydroxy epimers of progesterone.

20 mg of pure progesterone was dissolved in 100 ml absolute ethanol and suitable dilutions were made with absolute ethanol to give a series of solutions of known concentration. The optical density of these solutions was measured at 220, 240 and 260 mµ using quartz microcells in the Unicam S.P. 500 spectrophotometer, and the calibration curves shown opposite in Figure 3.A.3. constructed. It will be seen that over the range used the relationship of progesterone concentration to optical density is linear thus following the Beer-Lambert Law.

(3) Recovery of progesterone added to bovine plasma.

Pooled bullock plasma was obtained at the abattoir. To 500 ml aliquots of this plasma was added 5 µg of pure

TABLE 3.A.1.

RECOVERY OF PROGESTERONE ADDED TO 500 ml BOVINE CASTRATE MALE PLASMA.

| Amount Added in µg. | Amount Recovered in µg. | Percentage Recovery |
|---|---|---|
| Nil | Nil | Nil |
| 5.20 | 3.10 | 56.9% |
| 5.20 | 2.82 | 54.2% |
| 5.20 | 2.88 | 55.4% |
| 5.20 | 2.58 | 49.6% |
| 5.20 | 4.10 | 78.9% |
| S.D. = 10.17; S.E. = 4.55 | | Mean = 59.5% |

progesterone and the estimation as described on the flow sheet in the method section was carried out. Table 3.A.1. shows the quantities of progesterone which were obtained at the end of the procedure. It will be seen that no progesterone was detected in the blank sample and that the average recovery of the added progesterone was 59.5% ± 4.55 (S.E.). This compares favourably with the results obtained by Short (1958[b]) who recorded a mean recovery of 63% ± 2% (S.E.).

In an attempt to establish a basal level in bullock blood a further 2½ litres of pooled bullock plasma collected at slaughter was estimated as individual portions of 500 ml. Again there was no detectable progesterone in any of the individual 500 ml samples or in the 5 eluted samples re-pooled and rechromatographed as one sample. However, it was observed that with each sample and as in the original plasma blank the first chromatogram showed a spot which ran at the same Rf. as progesterone but which had no absorption peak at 240 millimicrons, was negative to the Zimmerman reagent, and disappeared when subjected to acetylation and rechromatography. The same type of contamination was seen on the first chromatogram with a

TABLE 3.A.2.

RECOVERY OF PROGESTERONE ADDED TO 1000 ml. ETHER.

| Amount Added in µg. | Amount Recovered in µg. | Percentage Recovery |
|---|---|---|
| 5.20 | 4.43 | 85.2% |
| 5.20 | 4.52 | 86.9% |
| 5.20 | 4.80 | 92.3% |
| 6.10 | 5.51 | 90.3% |
| 6.10 | 5.02 | 82.3% |
| 6.10 | 5.50 | 90.2% |
| S.D. = 3.42; S.E. = 1.40 | | Mean = 87.9% |

TABLE 3.A.3.

RECOVERY OF PROGESTERONE FROM PAPER AFTER CHROMATOGRAPHY AND ELUTION.

| PROGESTERONE | | |
|---|---|---|
| Amount Added in µg. | Amount Recovered in µg. | Percentage Recovery |
| 5.20 | 5.00 | 96.2% |
| 5.20 | 4.95 | 95.2% |
| 5.20 | 4.60 | 88.5% |
| 6.10 | 5.65 | 92.6% |
| 6.10 | 6.00 | 98.4% |
| 6.10 | 5.48 | 89.8% |
| S.D. = 3.84: S.E. = 1.72 | | Mean = 93.4% |

number of cow plasma samples. The exact nature of this material is not known but was thought to be a fairly polar lipid material from the plasma.

(4) Recovery of progesterone added at various steps in the procedure.

In view of the low recovery from bovine plasma information was sought as to where the losses of progesterone were occurring. To this end the procedure was broken down into steps and known quantities of progesterone added at various points after which the estimation was carried out to completion:

I. RECOVERY OF PROGESTERONE ADDED TO 1000 ML ETHER. Table 3.A.2. shows the results obtained when 5 µg of progesterone was added to 1000 ml of ether and it will be seen that 87.9% ± 1.4% (S.E.) of the added progesterone was recovered.

II. RECOVERY OF PROGESTERONE APPLIED TO PAPER. Table 3.A.3. shows the results obtained when 5 µg of progesterone was applied directly to Whatman No.20 chromatography paper and the procedure, chromatography, elution and spectrophotometry, carried to completion. In this case the average recovery of the added material was 93.4% ± 3.5%.

TABLE 3.A.4.

COMPARISON BETWEEN RECOVERIES OF PROGESTERONE AND RADIO PROGESTERONE FROM 500 ml BULLOCK PLASMA.

(4.2 µg "Organon" progesterone in 1 ml. ethanol and 1100 counts of progesterone - 4 - C14 in 0.2 ml Benzene).

| Sample | Progesterone recovered | Counts/min recovered | Percentage Recovery | |
|---|---|---|---|---|
| | | | Progesterone | Radio-progesterone |
| 1 | 1.75 | 430 | 39 | 41 |
| 2 | 2.75 | 723 | 61 | 66 |
| 3 | 2.30 | 591 | 51 | 54 |
| 4 | 1.81 | 482 | 40 | 44 |
| 5 | 2.70 | 702 | 62 | 64 |
| | | Mean: | 51 | 54 |

TABLE 3.A.5.

COMPARISON BETWEEN RECOVERIES OF PROGESTERONE AND RADIO PROGESTERONE FROM 1000 ml ETHER.

(4.5 µg "Organon" progesterone in 1 ml. ethanol and 1100 counts of progesterone - 4 - C14 in 0.2 ml Benzene).

| Sample | Progesterone recovered | Counts/min recovered | Percentage Recovery | |
|---|---|---|---|---|
| | | | Progesterone | Radio-progesterone |
| 1 | 4.15 | 998 | 92 | 91 |
| 2 | 4.10 | 1051 | 91 | 95 |
| 3 | 3.70 | 902 | 82 | 81 |
| 4 | 3.72 | 976 | 82 | 88 |
| 5 | 3.75 | 1002 | 83 | 91 |
| 6 | 4.00 | 1011 | 89 | 92 |
| | | Mean: | 87 | 90 |

TABLE 3.A.6.

RECOVERY OF RADIO-PROGESTERONE ONLY FROM PAPER FOLLOWING CHROMATOGRAPHY AND ELUTION.

| R A D I O - P R O G E S T E R O N E | | |
|---|---|---|
| Counts/min added | Counts/min recovered | Percentage recovery |
| 1030 | 942 | 91.5% |
| 1030 | 986 | 95.7% |
| 1030 | 988 | 95.9% |
| 1030 | 970 | 94.2% |
| 1030 | 946 | 91.8% |
| 1030 | 1004 | 97.5% |
| S.D. = 2.40; S.E. = 1.07 | Mean = | 94.4% |

The comparable figure for non radio-active progesterone was 93.4% $\pm$ 1.7 (S.E.) as shown in TABLE 3.A.3.

From the above results it will be seen that the greatest loss occurs when the plasma is extracted with ether as subsequent to this good yields are obtained.

(5) The Use of Progesterone - 4 - C14 as an internal marker.

In view of the wide range of recoveries (78.9% to 49.6%) (Table 3.A.1.) obtained it was felt that it would be desirable in this study, which involves the detection of small fluctuations in very low levels, to have a measure of individual recoveries. It was therefore decided to repeat the above recovery experiments using C14 labelled progesterone, firstly, to confirm the results obtained and, secondly, to determine if in fact there was good agreement between the recoveries of progesterone and radio-progesterone (progesterone - 4 - C14) in individual samples. The recoveries of the two types of progesterone from plasma, ether and paper are shown in Tables 3.A.4., 3.A.5., and 3.A.6., respectively. It will be seen that in all cases the recovery of the progesterone closely parallels that of the progesterone - 4 - C14. This confirms the previous recovery experiments and would indicate that the use of progesterone - 4 - C14 as an internal marker is justified.

Further information on the structure and preparatory work of progesterone - 4 - C14 is given in the appendix.

## DISCUSSION OF METHODS

The method used in the present study appeared to be well suited to the estimation of progesterone in bovine plasma in that a very small amount of steroid could be measured in a large volume of plasma. The incorporation of a radioactive marker to calculate individual recovery rate was particularly useful as it was found that recoveries of added progesterone in addition to being low (in the order of 50%) also varied between individual samples with a range of 40% to 70% recovery. Another advantage of having an internal marker was that in the event of additional purification steps being necessary with an individual sample, then the exact recovery was known for this particular sample. From the results obtained in recovery experiments it is apparent that the losses of added progesterone occurred during the ether extraction of the plasma. This is in agreement with the finding of Short (1958[a]) who found, in addition, a marked species variation. It has been shown by Eik-Nes, Schellman, Lumry and Samuels (1954) that a number of different steroids are in reversible combination with proteins in solution. Also, Wesphal, Firshchein and Pearce (1955) have shown that 90% of progesterone - 4 - C14 added to

human albumin migrated with the protein during electrophoresis. However, it would appear that with many steroids the link with protein is easily broken when the plasma is extracted with organic solvents. (Bibile, 1953 and Bush, 1955). However, Bischoff, Stauffer and Gray (1954) have indicated that the less polar steroids (which include progesterone) and the phenolic steroids (which include oestrogen) may be more firmly bound to proteins than the corticosteroids. However, Hooker and Forbes (1949) estimated that progesterone was 90% free in plasma. The observation that recovery of both progesterone and progesterone - 4 - C14 added to bovine plasma was lower when gel formation occurred during ether extraction suggests that in these cases a mechanical trapping of either the progesterone or of the ether or both may be partly responsible for the low recoveries in these cases.

The method for estimating progesterone in plasma as used in these studies was not ideal due to the large volumes of plasma (500 to 800 ml) required to yield a sufficient amount of progesterone for accurate measurement. Consequently, very large volumes of ether had to be handled both during initial purification of solvents and during repeated extraction of the plasma samples. As a result of

the large amount of plasma extracted, the amount of contaminating lipid material was proportionately large, and this led to difficult separation during the initial chromatography step. The use of the acetylating step, in addition to assisting in the removal of impurities, also made the estimation very much more specific for progesterone. (Bush, 1961).

It would appear therefore that a method with an increased sensitivity would be of immense value in the estimation of bovine plasma samples as this would cut down both the volume of plasma and the volume of solvents used and consequently the time taken for the estimation. In addition it would then be possible in the individual animal to collect a greater number of plasma samples at more frequent intervals than was possible in this present study. One such improvement in sensitivity is that reported by Touchstone and Murawec (1960) of an increased fluorescence of progesterone in sulphuric acid when subjected to prior treatment with sodium hydroxide. Short and Levett (1962) have recently utilised this finding as the basis of a method of measuring the low concentration of progesterone during the menstrual cycle in women using only small volumes of blood. Considerable difficulty was experienced with

fluorescence of the chromatography paper blank during the estimation but in spite of this the method produced results of considerable interest.

Bush (1961) has suggested an improvement in sensitivity by developing an isotopic method for progesterone determination. He suggested that ketalation of progesterone with labelled ethanedithiol or ethylene glycol could be carried out, followed by a determination of the radio activity in the sample.

Zander (1962) has suggested that if the dinitrophenylhydrazine or thiosemicarbazide derivations of progesterone were made with C14 labelled reagents a quantitative method for the estimation of progesterone could be developed. He also suggested a method involving the chemical or enzymatic reduction of the ketone group at C-20 followed by acetylation with C14 acetic anhydride and measurement of the radio-activity of the isolated derivative.

The method described by Rowlands and Short (1959) for progesterone estimation in luteal tissue was found to be suitable, but the recoveries of progesterone obtained in this study were somewhat variable. The addition of

an internal marker of progesterone - 4 - C14 was found to be an advantage. The recoveries obtained were good, with a range of 60 to 90%; slightly lower than those obtained by Rowlands and Short (1959) but higher than those obtained by Stormshak, Hunt and Erb (1961), using a different method but with an internal marker of progesterone - 4 - C14.

## SECTION 3 - RESULTS AND DISCUSSION

### B. PROGESTERONE LEVELS IN THE CYCLING COW.

In this section the results obtained in cycling cows are presented in the following order:

(1) Progesterone concentration in peripheral blood of cows during the oestrous cycle.

(2) Progesterone concentration after removal of the corpus luteum in the cow at mid-cycle.

(3) Progesterone concentration after bilateral ovariectomy in the cow at mid-cycle.

(4) Metabolism of injected progesterone in ovariectomised cows.

(5) Progesterone in the body fat of cows at mid-cycle.

(6) Progesterone concentration in milk.

## B. EXPERIMENTAL RESULTS

### (1) PLASMA PROGESTERONE CONCENTRATION DURING THE OESTROUS CYCLE IN THE COW.

Selection of cows for the series: Adult Ayrshire cows were purchased from local farms for the purpose of following progesterone levels in peripheral blood during the oestrous cycle. Before purchasing, an investigation into the animals' breeding history and physical condition was carried out on the farm to ascertain that each cow was compatible with normality. Particular attention was paid to the reproductive tract to make sure that no gross physical abnormality was present and that the animals were suitable for periodic rectal examination.

During the winter months the subjects were kept in a byre along with other normal cows. In order to detect oestrus they were turned into a small covered yard along with at least two other adult cows and observed thrice daily for signs of oestrus.

During the summer and spring the subjects were kept in a small paddock reserved for a number of normal

cows and they were again observed three times daily for signs of oestrous behaviour. The following list records the oestrous signs noted in the six subjects. The first two signs were observed in all the animals while the remainder were variable in occurrence:

1. Standing steadily for other cows to mount.
2. Lateral movement of the tail head to expose external genitalia.
3. Rhythmical pulsation of the lumbar muscles.
4. Bellowing together with general restlessness.
5. Occasional appearance of mucus from the vulva.

In addition each cow was examined by rectal palpation for uterine turgidity on the day of oestrus. Subsequently, ovarian palpation was carried out every second day in order to determine the growth of the corpus luteum. In all subjects a structure resembling a corpus luteum could be palpated in one of the ovaries by the 6th day post oestrus. In addition in another series of experiments involving some of these animals where the corpus luteum was surgically removed at a known time after oestrus it was evident from the size of the gland that the predicted time of oestrus was correct.

TABLE 3.B.1.

SUMMARY OF HISTORIES OF THE SIX COWS USED FOR PLASMA PROGESTERONE DETERMINATION DURING THE OESTROUS CYCLE.

| C O W | A | B | C | D | E | F |
|---|---|---|---|---|---|---|
| Age in years | 5½ | 3½ | 10 | 8 | 2½ | 4 |
| Number of previous parturitions | 2 | 2 | 7 | 5 | 1 | 2 |
| Month of sampling | Mar. | June | Jan. | Feb. | May | Aug. |
| Lactating | yes | yes | yes | yes | yes | no |
| Interval since last parturition in months | 2 | 4 | 4 | 5 | 3 | 6 |
| Weight in lbs. | 957 | 1156 | 832 | 881 | 1073 | 1008 |

A summary of the histories of the six cows used as experimental animals is shown in Table 3.B.1.

A blood sample was collected from each subject as soon as possible after oestrus was detected and on subsequent days of the cycle between the hours of 9 a.m. and 10 p.m. None of the animals appeared to be affected by the collection of approximately 1200 ml of blood which was carried out quickly and with as little excitement as possible.

The day of oestrus was taken as day one of the cycle and within the series three cows had 21 day cycles and three had 23 day cycles. These are all within the limits of the normal cycle length (Asdell, 1946).

On examination of the six graphs it will be seen that there is a considerable variation between cows in the levels reached during mid-cycle, but good agreement in the low values seen immediately pre and post oestrus. The basic pattern which emerges in each of the six animals is as follows:-

There is a low level of oestrus followed by a sharp rise until day ten when there is a plateau formation

until day 17 at which point the level falls to the oestrous level again. A description incorporating the salient features of the results for the individual cow are given immediately following the appropriate figure and table.

FIGURE 3.B.1.

PLASMA PROGESTERONE CONCENTRATION DURING CYCLE

COW - A.

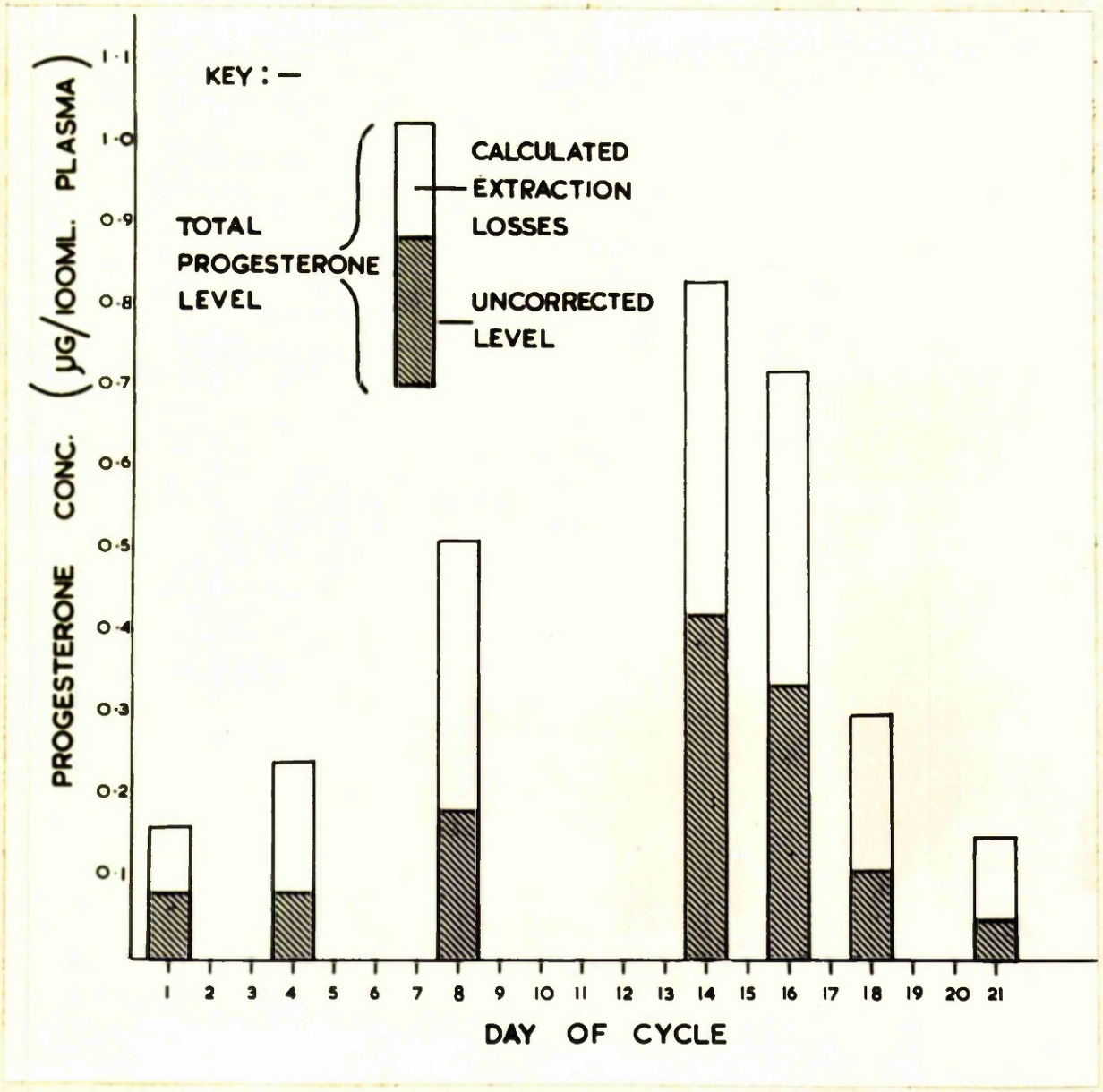


TABLE 3.B.2.

PLASMA PROGESTERONE CONCENTRATION DURING OESTROUS CYCLE

- COW A.

(2 extracts with an equal volume of ether).

| Day of Cycle | Volume of Plasma (ml) | Progesterone level (µg/100 ml plasma) | Recovery of Radioactivity (%) |
|---|---|---|---|
| 1 | 500 | 0.16 | 50 |
| 4 | 500 | 0.24 | 33 |
| 8 | 600 | 0.51 | 35 |
| 14 | 600 | 0.83 | 51 |
| 16 | 600 | 0.72 | 47 |
| 18 | 500 | 0.30 | 35 |
| 21 | 500 | 0.15 | 33 |
| | | Mean = | 41 |

## (a) PLASMA PROGESTERONE LEVELS IN COW A. DURING THE OESTROUS CYCLE.

Figure 3.B.1. shows the results obtained for Cow A in a pilot experiment to determine the stage of the cycle at which it was possible to detect progesterone and to establish the basic pattern of events. It will be seen that there is a uniform rise and fall of progesterone concentration between the successive days of oestrus, in this subject day 1 and 21. The peak level of progesterone occurred at about mid cycle and reached a maximum level of 0.8 µg/100 ml plasma. The recovering rates of progesterone - 4 - C14 were uniformly low in all the samples and this was probably due to the fact that only two ether extracts were used.

FIGURE 3.B.2.

PLASMA PROGESTERONE CONCENTRATION DURING CYCLE

- COW B.

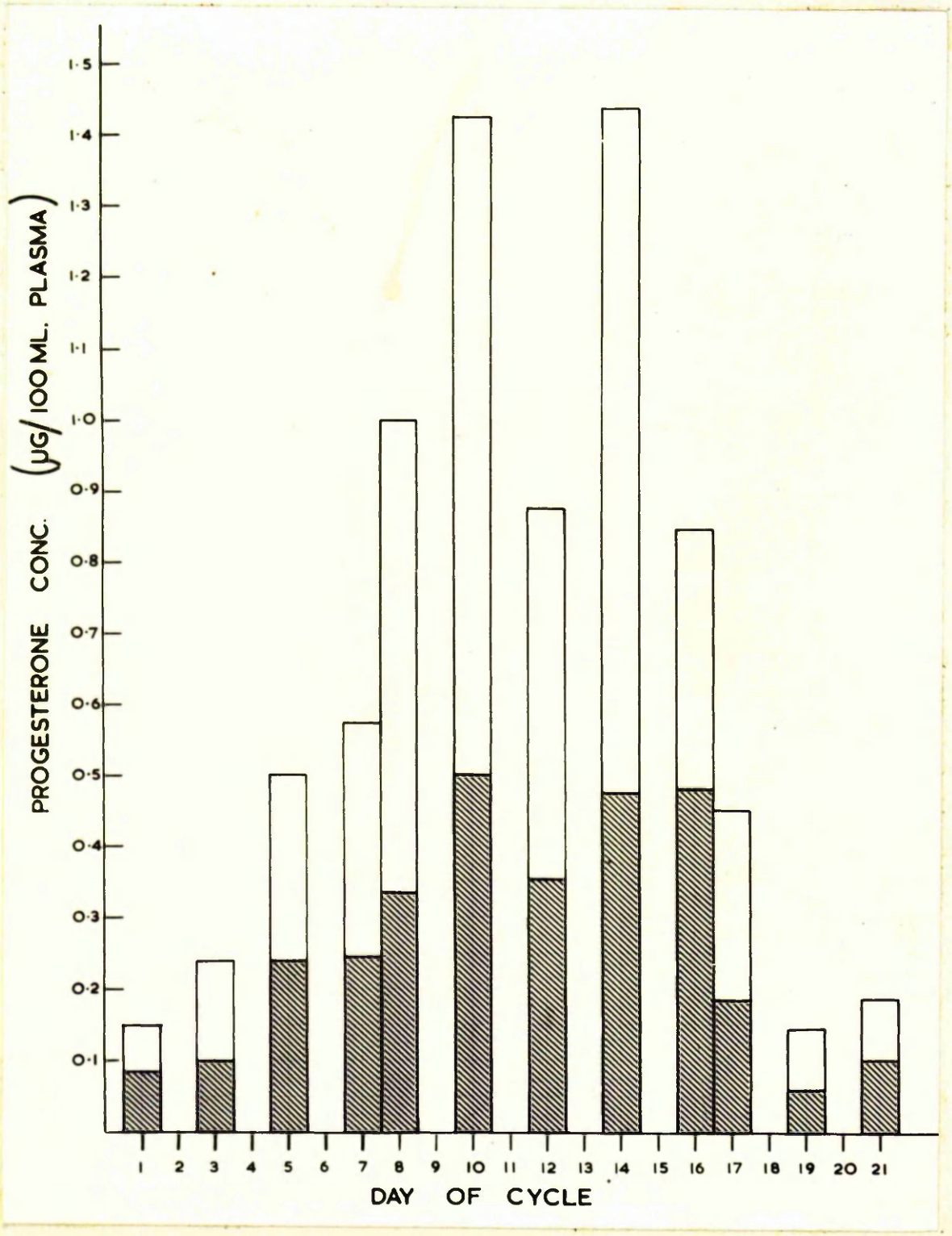


TABLE 3.B.3.

PLASMA PROGESTERONE CONCENTRATION DURING OESTROUS CYCLE

- COW B.

(2 extracts with an equal volume of ether).

| Day of Cycle | Volume of Plasma (ml) | Progesterone level (µg/100 ml plasma) | Recovery of Radioactivity (%) |
|---|---|---|---|
| 1 | 500 | 0.15 | 53 |
| 3 | 520 | 0.24 | 44 |
| 5 | 550 | 0.50 | 47 |
| 7 | 610 | 0.57 | 40 |
| 8 | 600 | 1.00 | 33 |
| 10 | 600 | 1.43 | 35 |
| 12 | 600 | 0.88 | 40 |
| 14 | 640 | 1.44 | 33 |
| 16 | 620 | 0.84 | 58 |
| 17 | 650 | 0.45 | 40 |
| 19 | 640 | 0.14 | 33 |
| 21 | 700 | 0.19 | 51 |
| | | | Mean = 42.3 |

## (b) PLASMA PROGESTERONE LEVELS IN COW B. DURING THE OESTROUS CYCLE.

Figure 3.B.2. shows the results obtained for Cow B from which samples were collected at more frequent intervals. It will be seen that the level rises quite sharply from days 1 to 8 and thereafter the levels appear to fluctuate, reaching at times the highest recorded in this series, before falling to almost zero at day 21. The difference in levels on days 19 and 21 are not significant as they are both bordering on the lower limits of sensitivity of the method. The recovery rates are again low due to the use of only two extracts of ether and in certain cases, the need for rechromatography in order to effect a clean separation.

FIGURE 3.B.3.

PLASMA PROGESTERONE CONCENTRATION DURING CYCLE

- COW C.

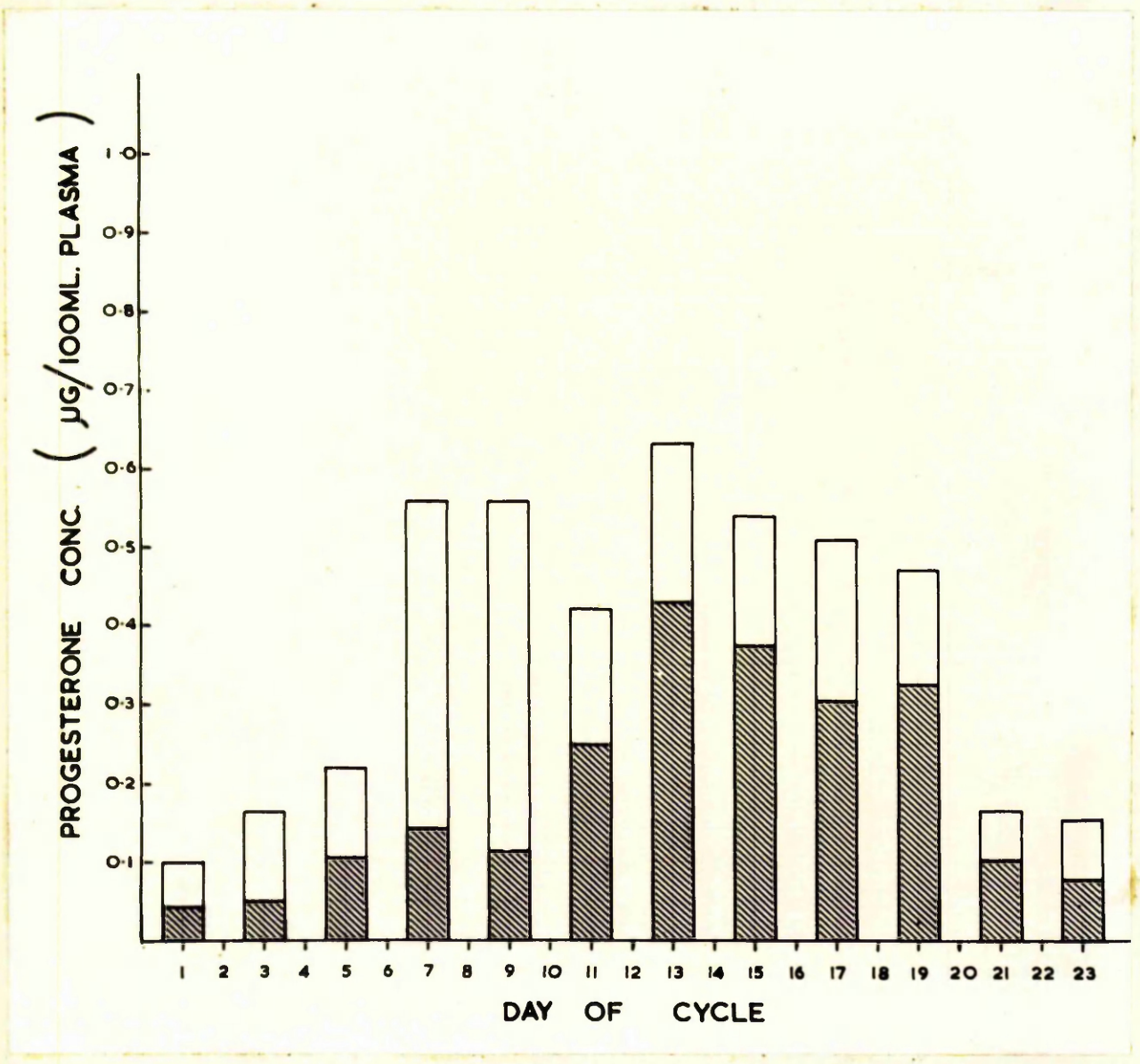


TABLE 3.B.4.

PLASMA PROGESTERONE CONCENTRATION DURING OESTROUS CYCLE

- COW C.

(4 extracts with an equal volume of ether).

| Day of Cycle | Volume of Plasma (ml) | Progesterone level (µg/100 ml plasma) | Recovery of Radioactivity (%) |
|---|---|---|---|
| 1 | 500 | 0.10 | 38 |
| 3 | 500 | 0.16 | 31 |
| 5 | 570 | 0.22 | 47 |
| 7 | 560 | 0.56 | 25 |
| 9 | 610 | 0.56 | 21 |
| 11 | 620 | 0.42 | 60 |
| 13 | 650 | 0.63 | 68 |
| 15 | 640 | 0.54 | 69 |
| 17 | 680 | 0.51 | 59 |
| 19 | 660 | 0.47 | 69 |
| 21 | 600 | 0.16 | 63 |
| | | | Mean = 50 |

## (c) PLASMA PROGESTERONE LEVELS IN COW C. DURING THE OESTROUS CYCLE.

Gel formation in the samples taken at the beginning of the cycle was responsible for the low recoveries seen at this time. The maximum level at mid cycle was low, reaching only 0.5 µg/100 ml plasma, while the curve did not show signs of regression until day 19 to give a cycle of 23 days duration. This cow at 10 years of age, was the oldest animal in the series.

FIGURE 3.B.4.

PLASMA PROGESTERONE CONCENTRATION DURING CYCLE

- COW D.

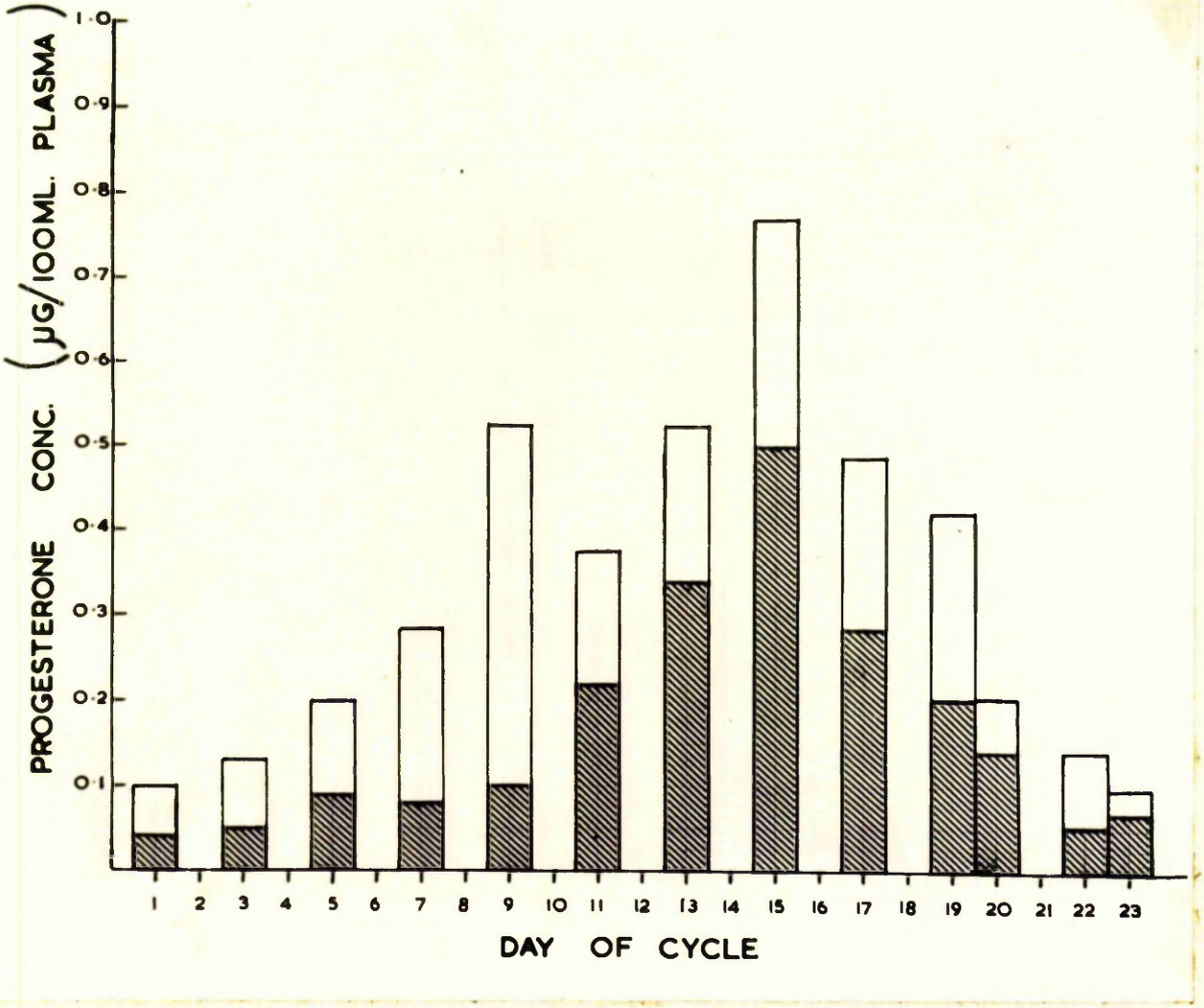


TABLE 3.B.5.

PLASMA PROGESTERONE CONCENTRATION DURING OESTROUS CYCLE

- COW D.

(4 extracts with an equal volume of ether).

| Day of Cycle | Volume of Plasma (ml) | Progesterone level (µg/100 ml plasma) | Recovery of Radioactivity (%) |
|---|---|---|---|
| 1 | 570 | 0.10 | 61 |
| 3 | 590 | 0.13 | 45 |
| 5 | 580 | 0.20 | 48 |
| 7 | 510 | 0.28 | 69 |
| 9 | 640 | 0.52 | 17 |
| 11 | 600 | 0.37 | 26 |
| 13 | 630 | 0.52 | 63 |
| 15 | 650 | 0.77 | 45 |
| 17 | 630 | 0.48 | 58 |
| 19 | 690 | 0.41 | 48 |
| 20 | 640 | 0.30 | 70 |
| 22 | 600 | 0.14 | 27 |
| 23 | 610 | 0.12 | 60 |
| | | | Mean = 49 |

## (d) PLASMA PROGESTERONE LEVELS IN COW D. DURING THE OESTROUS CYCLE.

This animal with a level of 0.77 µg/100 ml plasma was lower than average for the peak level at mid cycle. The recovery rates of progesterone - 4 - C14 were low at the beginning of the cycle again due to gel formation in the plasma. Both cow C and cow D tended to show a more gradual rise to a maximum than the other cows and both showed a longer cycle length - 23 days.

FIGURE 3.B.5.

PLASMA PROGESTERONE CONCENTRATION DURING CYCLE

- COW E.

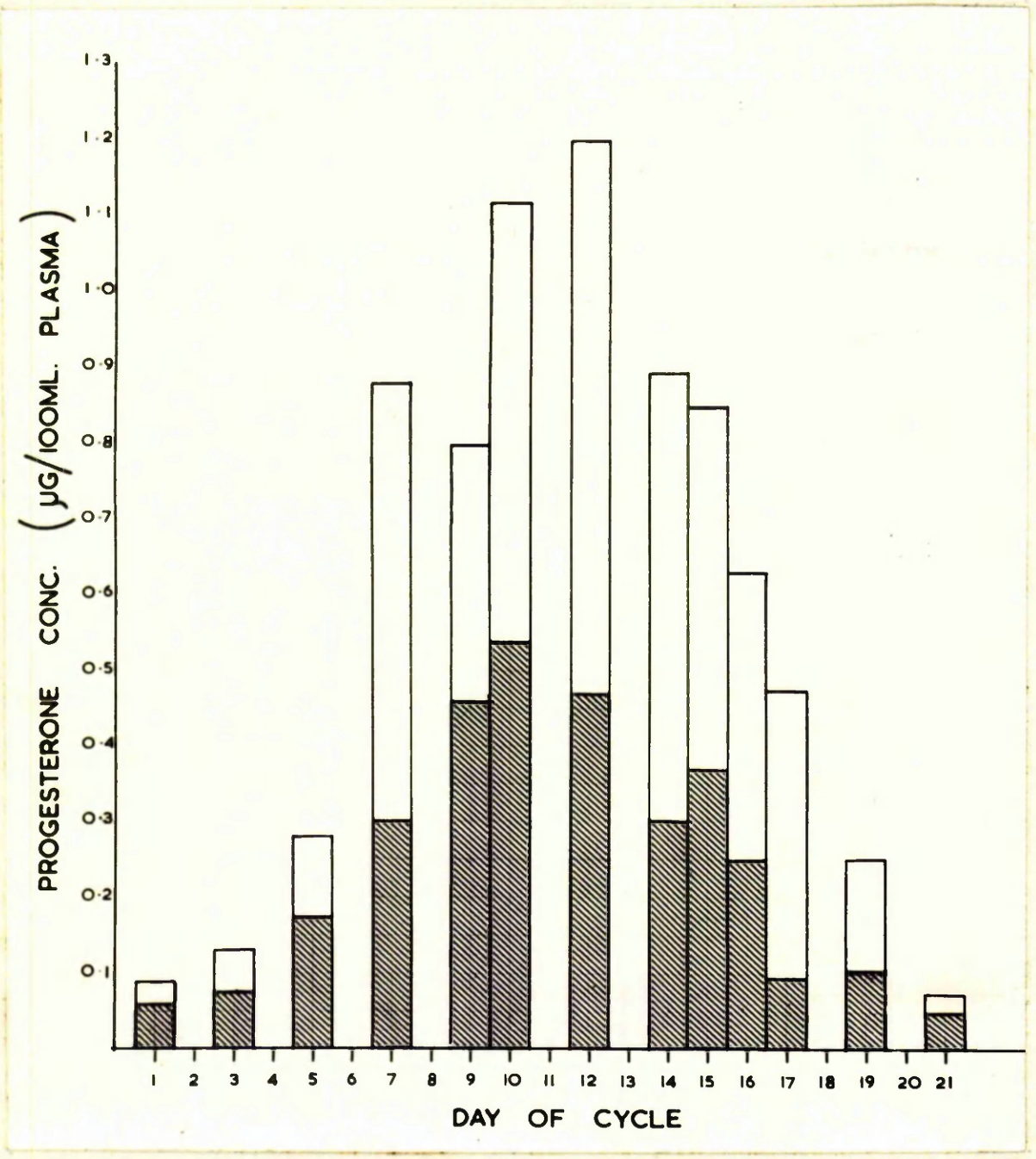


TABLE 3.B.6.

PLASMA PROGESTERONE CONCENTRATION DURING OESTROUS CYCLE

- COW E.

(4 extracts with an equal volume of ether).

| Day of Cycle | Volume of Plasma (ml) | Progesterone level (µg/100 ml plasma) | Recovery of Radioactivity (%) |
|---|---|---|---|
| 1 | 550 | 0.10 | 63 |
| 3 | 560 | 0.13 | 58 |
| 5 | 520 | 0.28 | 60 |
| 7 | 510 | 0.88 | 34 |
| 9 | 440 | 0.80 | 58 |
| 10 | 510 | 1.12 | 48 |
| 12 | 530 | 1.21 | 39 |
| 14 | 590 | 0.89 | 33 |
| 15 | 470 | 0.85 | 44 |
| 16 | 570 | 0.63 | 40 |
| 17 | 590 | 0.47 | 20 |
| 19 | 660 | 0.25 | 42 |
| 21 | 600 | 0.07 | 49 |
| | | Mean = | 45 |

## (e) PLASMA PROGESTERONE LEVELS IN COW E. DURING THE OESTROUS CYCLE.

The results for Cow E demonstrate an unusually sharp rise in progesterone concentration early in the cycle. It will be seen that a level of 0.88 µg/100 ml plasma is reached as early as day 7 while a fall in concentration appeared to commence on day 15 to give a cycle of 21 days duration. The maximum level of 1.21 µg/100 ml occurred on day 12 of the cycle which was earlier than in most animals. This was unexpected as in fact samples were collected on consecutive days (14 to 17) in order to pinpoint the day showing maximum level. Recovery rates of progesterone - 4 - C14 were uniformly consistent during the whole cycle. A visual estimate of the progesterone levels during the cycle in Cow E is shown in Figure 3.B.10. (see page 116).

FIGURE 3.B.6.

PLASMA PROGESTERONE CONCENTRATION DURING CYCLE

- COW F.

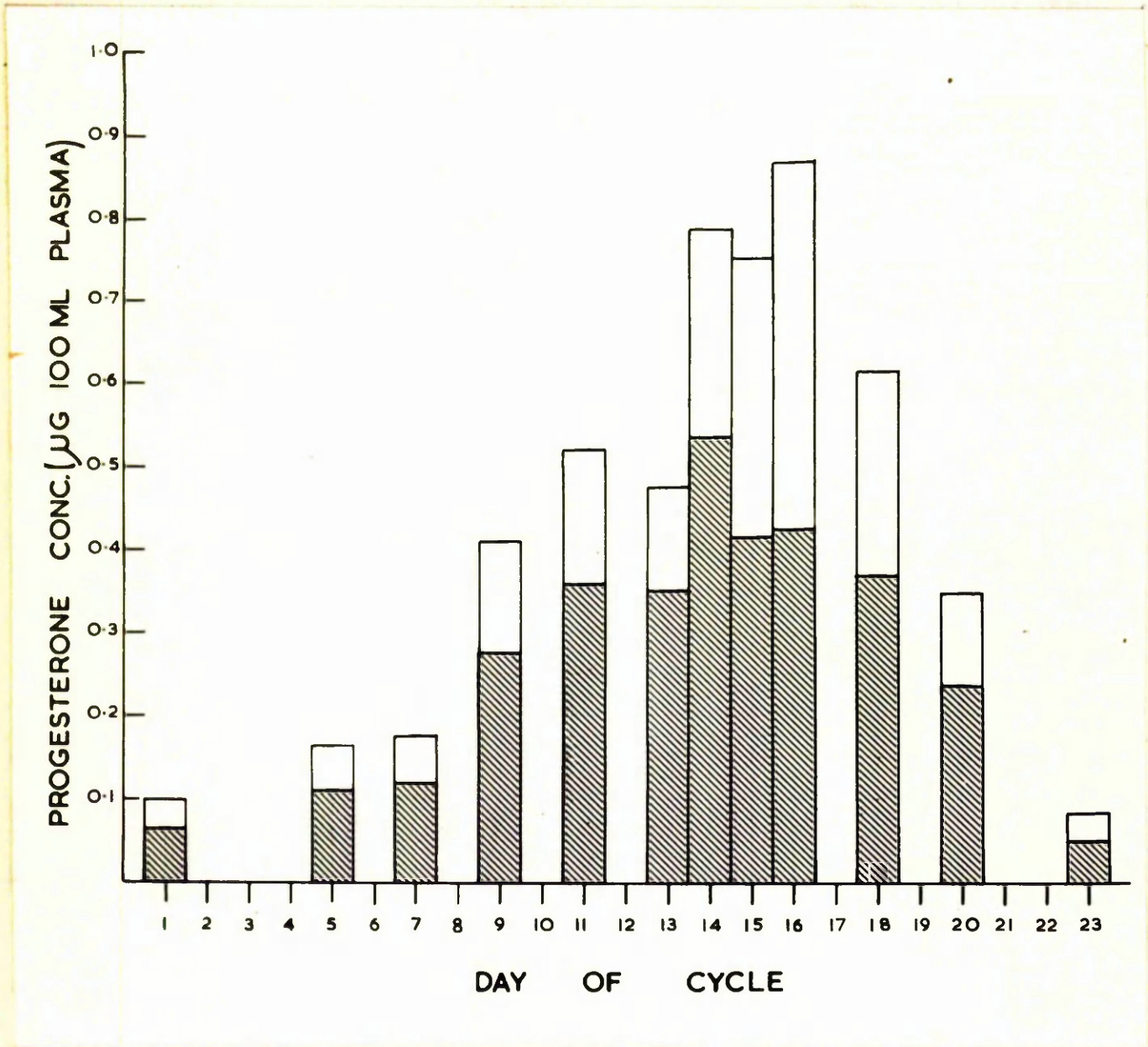


TABLE 3.B.7.

PLASMA PROGESTERONE CONCENTRATION DURING OESTROUS CYCLE

- COW F.

| Day of Cycle | Volume of Plasma (ml) | Progesterone level (µg/100 ml plasma) | Recovery of Radioactivity (%) |
|---|---|---|---|
| 1 | 530 | 0.10 | 65 |
| 5 | 510 | 0.16 | 70 |
| 7 | 520 | 0.17 | 70 |
| 9 | 560 | 0.41 | 68 |
| 11 | 630 | 0.52 | 69 |
| 13 | 610 | 0.47 | 75 |
| 14 | 550 | 0.79 | 68 |
| 15 | 620 | 0.75 | 55 |
| 16 | 560 | 0.86 | 49 |
| 18 | 620 | 0.61 | 60 |
| 20 | 640 | 0.34 | 69 |
| 23 | 600 | 0.08 | 55 |
| | | | Mean = 64 |

## (f) PLASMA PROGESTERONE LEVELS IN COW F. DURING THE OESTROUS CYCLE.

In Cow F it will be seen that there is a more gradual rise in progesterone concentration up to day 11 with a peak level at day 16. Regression of the curve does not occur until day 18 to give a cycle length of 23 days duration. Samples were collected consecutively on days 13 to 16 in an attempt to localise the day on which the maximum level occurred. From the results it is apparent that the levels on days 14, 15 and 16 were similarly high. The recovery rates of progesterone - 4 - C14 were uniformly high in all samples.

FIGURE 3.B.7.

COMPOSITE GRAPH OF PLASMA PROGESTERONE LEVELS

IN COWS A,B,C,D,E & F.

(Corrected for extraction losses)

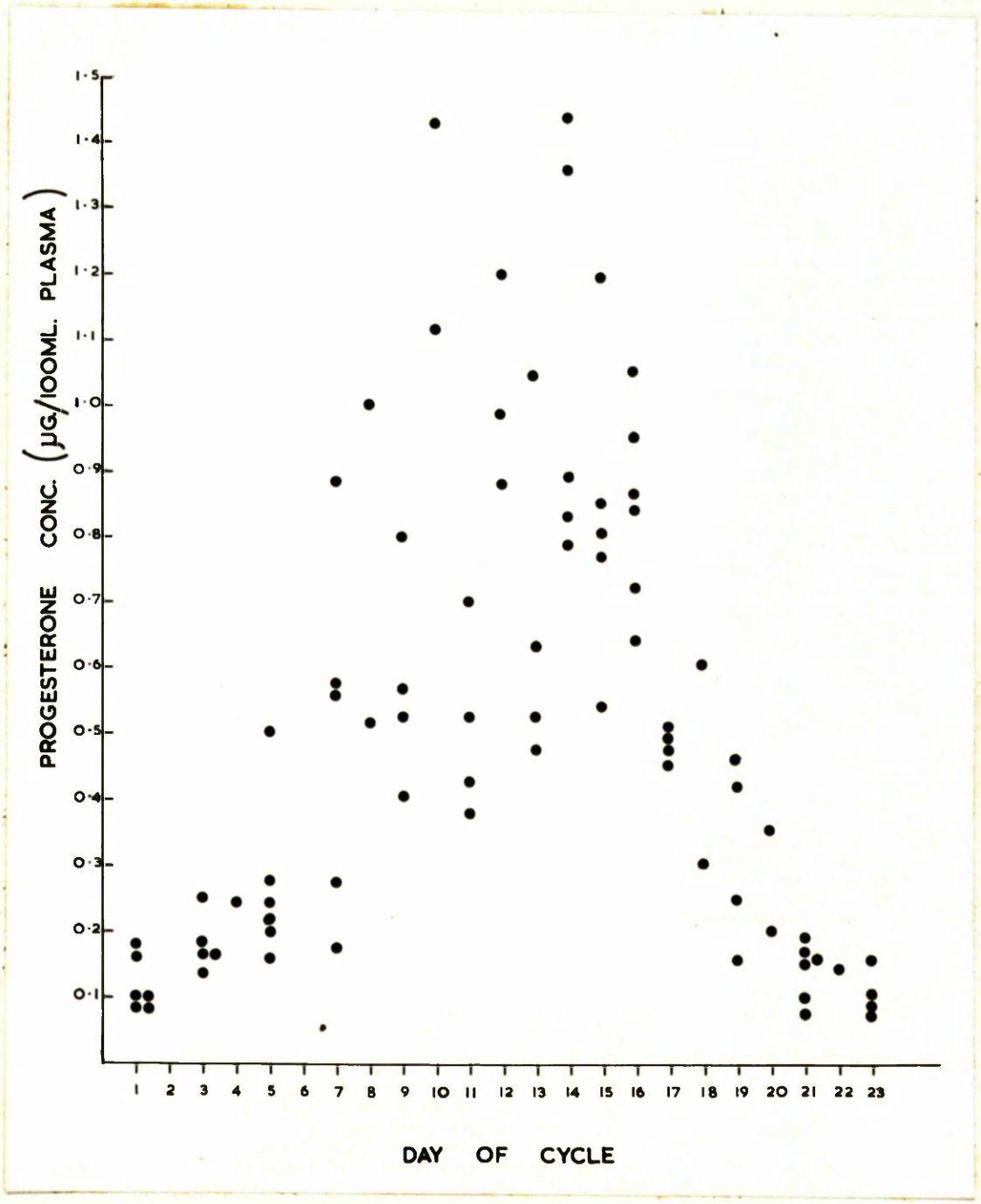


FIGURE 3.B.8.

COMPOSITE GRAPH OF PLASMA PROGESTERONE LEVELS

IN COWS A.B.C.D.E & F.

(Not corrected for extraction losses)

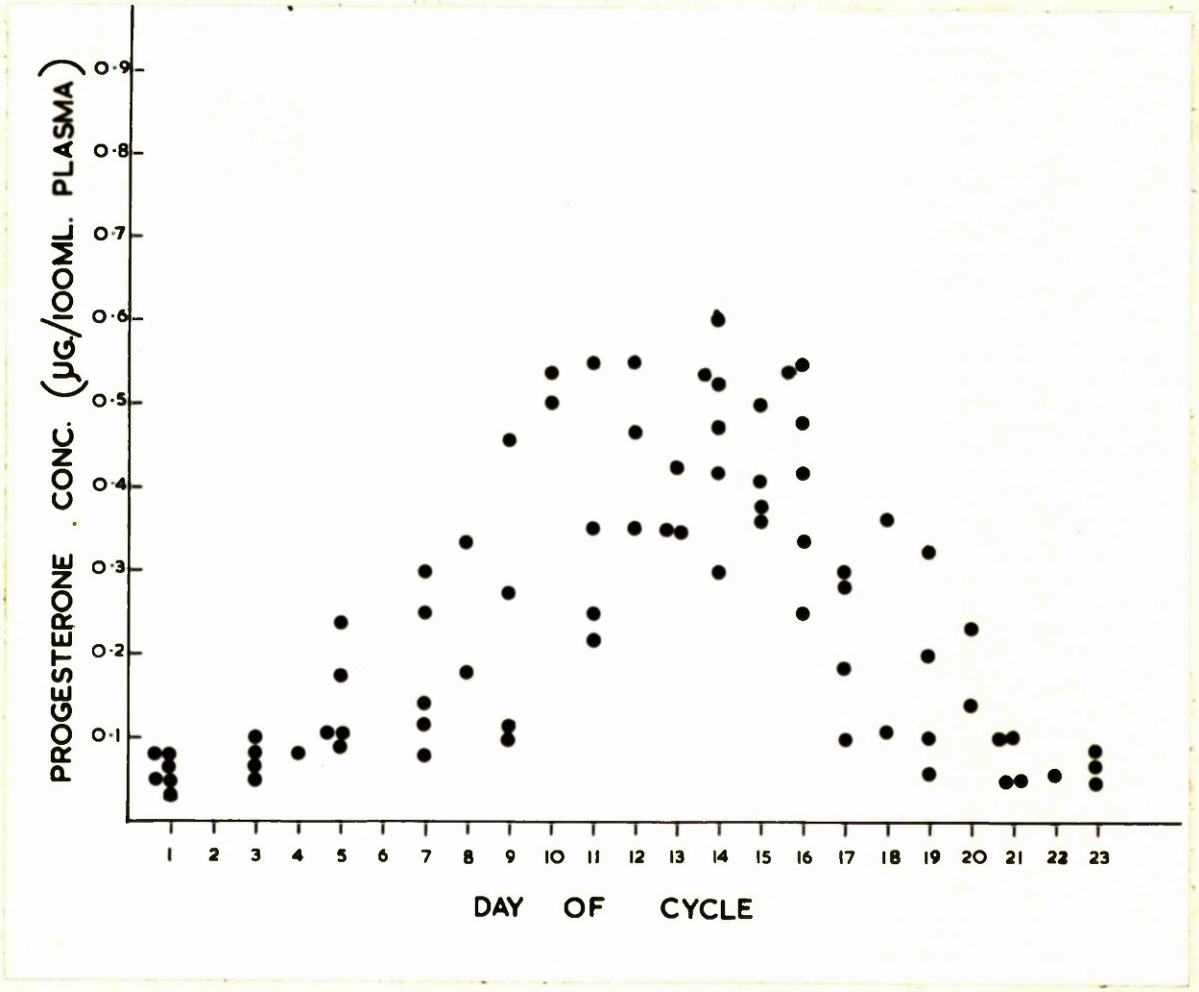
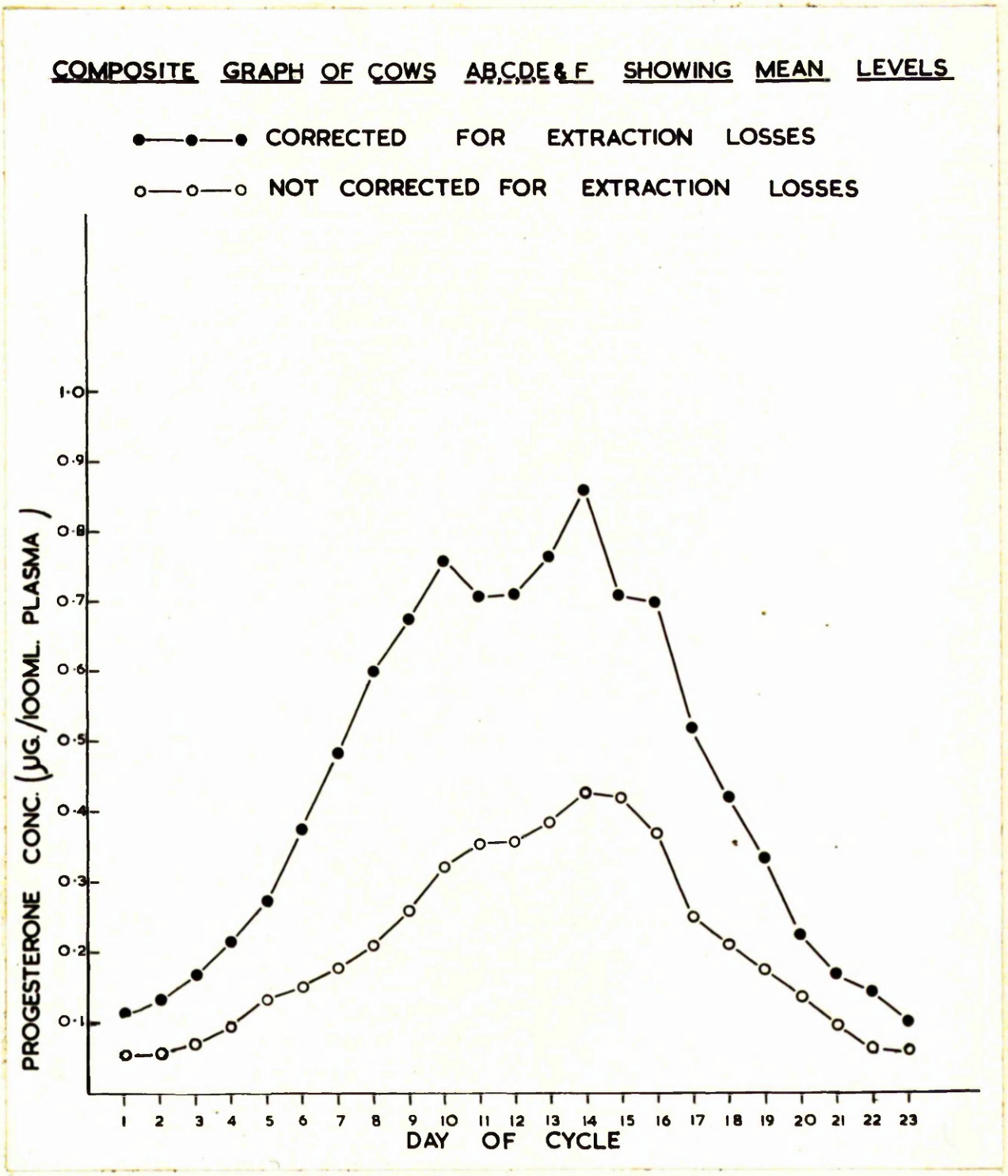


FIGURE 3.B.9.


COMPOSITE GRAPH OF COWS ABCDE & F SHOWING MEAN LEVELS
CORRECTED FOR EXTRACTION LOSSES
NOT CORRECTED FOR EXTRACTION LOSSES
PROGESTERONE CONC. (µG./100ML. PLASMA)
1·0
0·9
0·8
0·7
0·6
0·5
0·4
0·3
0·2
0·1
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23
DAY OF CYCLE

## COMPOSITE GRAPHS OF PLASMA PROGESTERONE LEVELS.

In order to demonstrate the range of values during the oestrous cycle, plasma progesterone levels of the six cows were plotted on the same graph. Figures 3.B.7. and 3.B.8. show the levels corrected for extraction losses and uncorrected respectively. It will be seen that there is greater variation in the corrected than in the uncorrected values. The daily mean values of progesterone concentration for the six cows during the cycle are shown in Figure 3.B.9. The shape of the curve for the corrected level closely resembles that of the uncorrected readings. It should be noted, however, that no allowance has been made for differences in cycle length.

The general pattern in the composite graph, Figure 3.B.9., would appear to be a gradual increase from the day of oestrus to about day five, followed by a more rapid rise to a plateau from day ten to day sixteen. The progesterone concentration then appears to fall to the day one level at a uniform rate, similar to that at which it originally rose to the plateau.

TABLE 3.B.8.

PLASMA PROGESTERONE CONCENTRATION DURING TWO SUCCESSIVE CYCLES IN THE SAME COW.

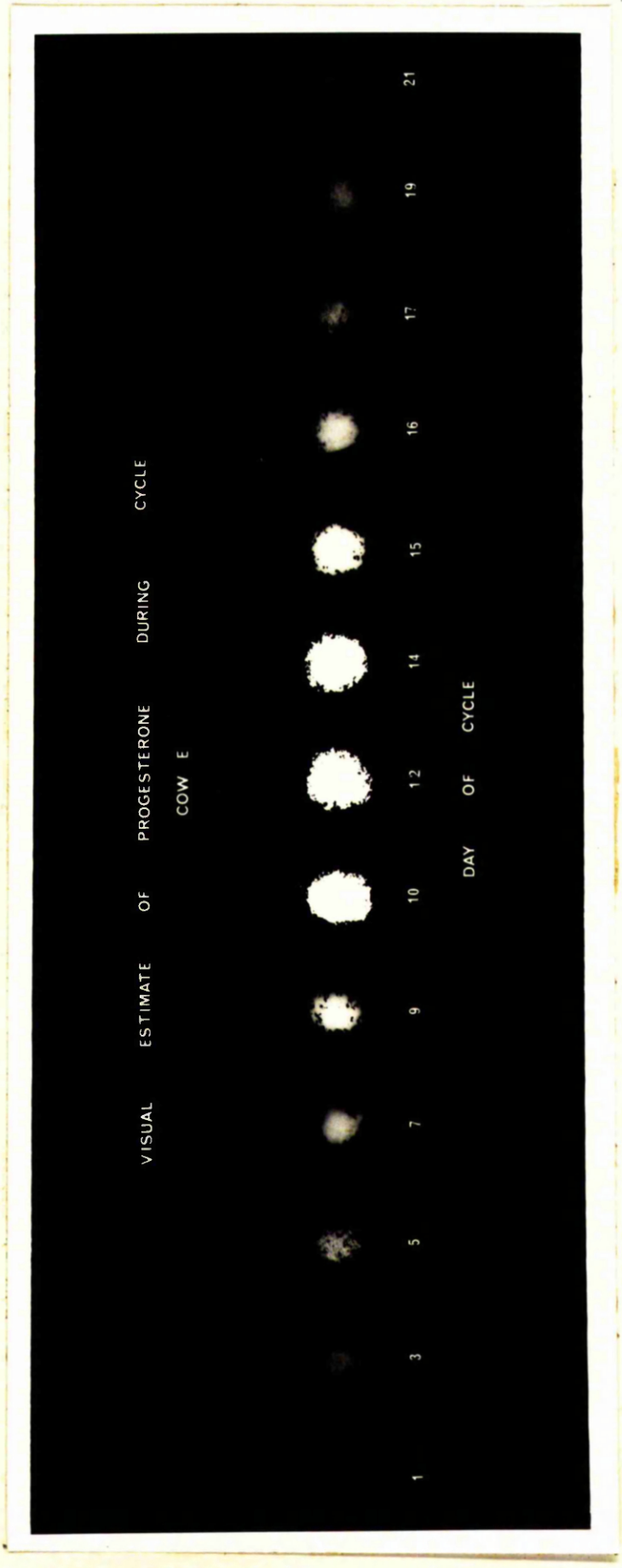
(Results expressed in µg/100 ml. plasma)

| Day of Sampling | C O W E. | | C O W F. | |
|---|---|---|---|---|
| | 1st cycle | 2nd cycle | 1st cycle | 2nd cycle |
| 11 | - | - | 0.52 | 0.70 |
| 12 | 1.21 | 0.98 | - | - |
| 13 | - | - | 0.47 | 1.05 |
| 14 | 0.90 | 1.35 | 0.79 | - |
| 15 | 0.85 | - | 0.75 | 1.18 |
| 16 | 0.63 | 1.05 | 0.86 | 0.95 |
| 17 | 0.47 | - | - | - |
| 18 | - | - | 0.61 | - |

## LEVELS OF PROGESTERONE ATTAINED BY THE SAME COW IN TWO SUCCESSIVE CYCLES.

In order to compare the levels attained during two successive cycles in the same cow, samples were collected from cows E and F during the cycles immediately following those just described. Only a small number of samples were taken on certain pre-selected days during mid cycle as it was considered that the removal of an excessive amount of blood following close on the previous experiment might be detrimental both to the health of the animal and to the validity of the results obtained. Accordingly, samples were collected on days 12, 14 and 16 in the case of cow E, and on days 11, 13, 15 and 16 in the case of cow F. The results are shown in Table 3.B.8. where it will be seen that in cow E the levels are of the same order but the peak has advanced from day 12 in the first cycle of 21 days duration, to day 14 in the second cycle of 22 days duration. In cow F the peak has changed from day 16 in the first cycle of 23 days duration, to day 15 in the second cycle of 22 days duration. It will be seen that in this subject the levels in the second cycle are higher than those in the first cycle.

FIGURE 3.B.10.


VISUAL ESTIMATE OF PROGESTERONE DURING CYCLE
COW E
1
3
5
7
9
10
12
14
15
16
17
19
21
DAY OF CYCLE

## DISCUSSION OF PLASMA PROGESTERONE LEVELS DURING THE OESTROUS CYCLE.

**Introduction:** In a very early study of the levels of "progestin" in the corpora lutea of sows, Kimura and Cornwell (1938) using a bio-assay method reported a rise and fall of "progestin" during the course of the 21 day cycle in the sow. Zander, Forbes, von Münsterman and Neher (1958) demonstrated a rise and fall in the total progesterone content of human corpora lutea between the time of ovulation and the subsequent menstrual period. It has also been shown during this time in women that pregnanediol, the main urinary metabolite of progesterone, also rises and falls. (Klopper, 1957; Brown, Klopper and Loraine, 1958).

In addition plasma levels of progesterone in women during the luteal phase of the cycle show a rise and fall with a peak level of about twice that seen during the follicular phase of the cycle. (Oertel, Weiss and Eik-Nes, 1959; Short, 1962; and Sommerville, Picket, Collins and Denyer, 1962).

The general pattern of progesterone concentration

seen during the oestrous cycle in the present study appeared to reflect remarkably well the physical increase in size of the corpus luteum in the cow during the cycle, an account of which is given by Hammond (1927). In addition the blood levels agreed well with histological changes in the cow corpus luteum reported by McNutt (1925), who showed that vascularization was complete by day 9, while lipoidal droplets appeared on day 14, and definite retrogression of luteal cells occurred on day 17 of the cycle. Also, Hansel (1959) found that the corpus luteum in the cow reached a maximum size on day 16 to 18 of the cycle then began to regress while, Hancock (1962), was able to detect clinically by rectal palpation that regression occurred about day 16 and that there was very rapid involution from day 18 onwards. Edgar et al. (1958) measured the progesterone concentration in ovarian vein blood in sheep during the 16 day oestrous cycle, and although there was a wide variation in levels between individuals the general trend was for the level to follow the growth and regression of the corpus luteum.

Information in the literature on plasma progesterone concentration in the cow is very limited and is concerned mostly with the levels found during pregnancy.

The maximum levels of progesterone in the six cows used in this study range from 0.63 to 1.44 µg/100 ml and are similar to these reported by Short (1958) in normal cows during pregnancy (0.8 µg to 1.0 µg/100 ml) and also in a series of cows which exhibited prolonged gestation but had normal levels - 0.65 to 1.35 µg/100 ml (Holm and Short, 1962). Using a different chemical method Bowerman and Melampy (1962) reported a much wider scatter of levels during normal pregnancy in the cows with a range of 0.00 µg to 2.13 µg/100 ml plasma. The only study of progesterone levels during the cycle in cows is that reported by Sullivan (1960) who measured progesterone levels in nine cows, but only on days 1, 7, 13 and 19 of the cycle, when he recorded mean levels in µg/100 ml plasma of 0.24, 0.56, 0.46 and 0.23, respectively. The corresponding figures in the present study are 0.11, 0.48, 0.78 and 0.34 µg/100 ml plasma which represents a much greater rise and fall. In addition, the level 0.11 seen on the day of oestrus in the present study is less than half that determined by Sullivan in spite of the fact that, using the present method, the day one level may be a slight over-estimate due to (a) Non-specific absorption from the paper at low optical density readings, and (b) The ever present danger of even minute traces of

progesterone surviving the rigorous washing up procedure.

There are a number of possible reasons as to why Sullivan's results differ from those reported in the present study. The most obvious difference is that of Sullivan's method which was quite involved; the final readings being based on the formation of the sulphuric acid chromogen of progesterone (Oertel, Weiss and Eik-Ness, 1959). In addition, he used only 350 ml of plasma per estimation, which is about half that used in the present study. This together with the fact that his recoveries of radio-active progesterone were low (12% to 55%) would tend to yield an amount of progesterone too small for accurate measurement. Another reason for the variation in findings is a possible difference in the criteria used for the detection of oestrus and the timing of the oestrous cycle, about which Sullivan gives very little information.

Lastly no mention was made of the washing up of glassware which is extremely important when estimating microgram quantities of steroid and could possibly have contributed to the discrepancies between the two sets of results.

In the present series the maximum mean level of

0.78 during the cycle is somewhat lower than the mean level seen in cows at both 14 and 16 days after insemination, both pregnant and non pregnant, as described in Section 3.C. However, this level in cycling cows may be disproportionately low due to the small number of animals (six cows) used in the present series and the fact that they may not all be truly representative of normality. For example, cows C and D showed both lower maximum and overall levels of progesterone than was seen in the other animals. It would be difficult in these cases to determine whether this was due to the fact that they were both much older animals than the rest, or the fact that they were both sampled at a time of the year (January and February) when reproductive activity of cows is known to be diminished.

A closer relation of cycling to inseminated cows is obtained by taking the mean of the maximum level attained during each cycle (cycle maximum) rather than the maximum mean daily level. The maximum levels seen during the cycle in cows A, B, C, D, E, and F are 0.83, 1.44, 0.63, 0.77, 1.21 and 0.79 µg/100 ml plasma, respectively. This gives a mean cycle maximum figure of 0.94 µg which is more comparable to the levels seen in inseminated cows prior to slaughter which had a range of means from 0.93 to 1.14 µg.

The curves representing the corrected and uncorrected levels which are shown in Figure 3.B.9 are more divergent during the first half of the cycle than during the second half. This indicates a lower recovery during the first half of the cycle which is probably a direct consequence of the increased tendency for the plasma to gel during this period and therefore to yield lower recovery rates.

TABLE 3.B.9.

PLASMA PROGESTERONE CONCENTRATION AFTER REMOVAL OF THE CORPUS LUTEUM PER VAGINUM ON DAY 12 OF CYCLE

COW B.

Corrected for extraction losses

| Time in minutes after removing corpus luteum | Volume of Plasma (ml) | Plasma Progesterone Concentration (µg/100 ml plasma) | Recovery of Radioactivity (%) |
|---|---|---|---|
| Control | 630 | 0.86 | 51 |
| 15 | 630 | 0.54 | 64 |
| 60 | 660 | 0.31 | 65 |
| 180 | 650 | 0.15 | 60 |
| 24 Hours | 640 | - | 52 |

Total weight of corpus luteum = 9.27 gms.

Progesterone content = 310.8 µg.

20B hydroxy progesterone = 33.4 µg.

TABLE 3.D.10.

PLASMA PROGESTERONE CONCENTRATION AFTER REMOVAL OF THE CORPUS LUTEUM PER VAGINUM ON DAY 12 OF CYCLE.

COW E.

Corrected for extraction losses

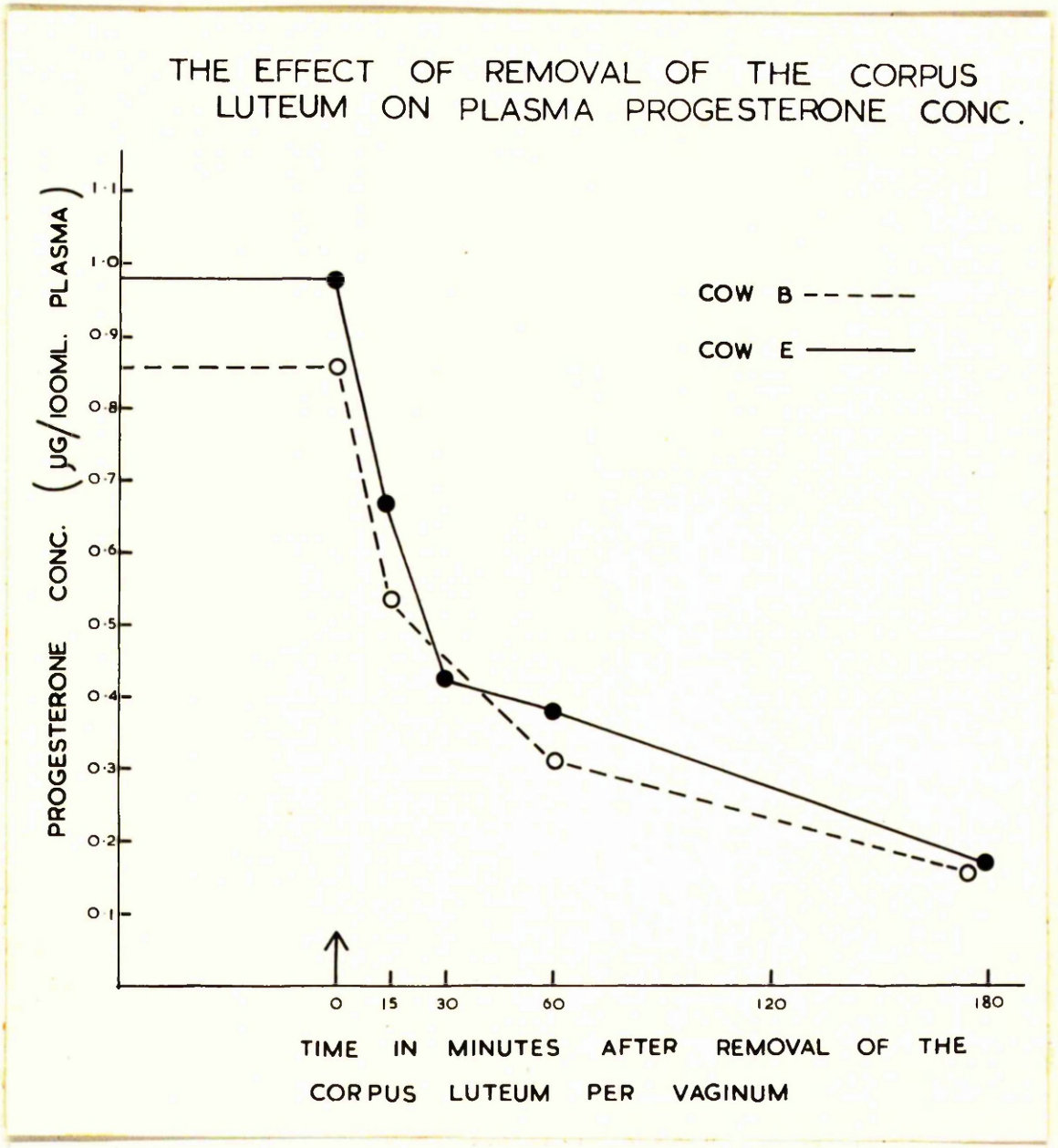
| Time in minutes after removing corpus luteum | Volume of Plasma (ml) | Plasma Progesterone Concentration (µg/100 ml plasma) | Recovery of Radioactivity (%) |
|---|---|---|---|
| Control | 630 | 1.08 | 35 |
| 14 | 600 | 0.67 | 39 |
| 30 | 610 | 0.39 | 64 |
| 60 | 610 | 0.38 | 40 |
| 180 | 600 | 0.17 | 60 |
| 24 Hours | 620 | 0.13 | 48 |

Total weight of corpus luteum = 5.82 gms.

Progesterone content = 224.5 µg.

20B hydroxy progesterone = 21.5 µg.

FIGURE 3.B.11.


THE EFFECT OF REMOVAL OF THE CORPUS LUTEUM ON PLASMA PROGESTERONE CONC.
PROGESTERONE CONC. (µG/100ML. PLASMA)
1·1
1·0
0·9
0·8
0·7
0·6
0·5
0·4
0·3
0·2
0·1
COW B
COW E
0
15
30
60
120
180
TIME IN MINUTES AFTER REMOVAL OF THE CORPUS LUTEUM PER VAGINUM

## (2) PLASMA PROGESTERONE CONCENTRATION AFTER REMOVAL OF THE CORPUS LUTEUM IN THE COW AT MID CYCLE.

In order to study the part played by the corpus luteum in maintaining the progesterone level in the blood, experiments were conducted in which the corpus luteum was removed at a time when the plasma progesterone level was at its highest, i.e. during the mid cycle plateau.

Cows B and E were selected for investigation and the corpus luteum in each subject was removed from the ovary per vaginum on day twelve of the cycle by the method described in section 2.C. page 47. The plasma progesterone concentration was measured immediately prior to, and at known times after removal of the gland, the results are shown in Table 3.B.9. and 3.B.10. for cows B and E respectively. The levels are also shown graphically in Figure 3.B.11. It will be seen that there is an initial rapid drop during the first fifteen minutes followed by a more gradual fall lasting some hours. In both cows there was no measurable progesterone 24 hours after surgery.

TABLE 3.B.11.

PLASMA PROGESTERONE CONCENTRATION AFTER REMOVAL OF BOTH OVARIES BY FLANK LAPAROTOMY ON DAY 12 OF CYCLE

COW B.

Corrected for extraction losses.

| Time in minutes after removing both ovaries | Volume of Plasma (ml) | Plasma progesterone concentration (µg/100 ml plasma) | Recovery of Radioactivity (%) |
|---|---|---|---|
| Control | 620 | 0.90 | 40 |
| 16 | 620 | 0.32 | 45 |
| 60 | 620 | 0.41 | 60 |
| 180 | 630 | 0.25 | 44 |
| 24 Hours | 610 | Trace | 52 |

Total weight of corpus luteum = 8.42 gms.
Progesterone content = 284.6 µg.
20B hydroxy progesterone = 10.3 µg.

TABLE 3.B.12.

PLASMA PROGESTERONE CONCENTRATION AFTER REMOVAL OF BOTH OVARIES PER VAGINUM ON DAY 14 OF CYCLE

COW E.

Corrected for extraction losses

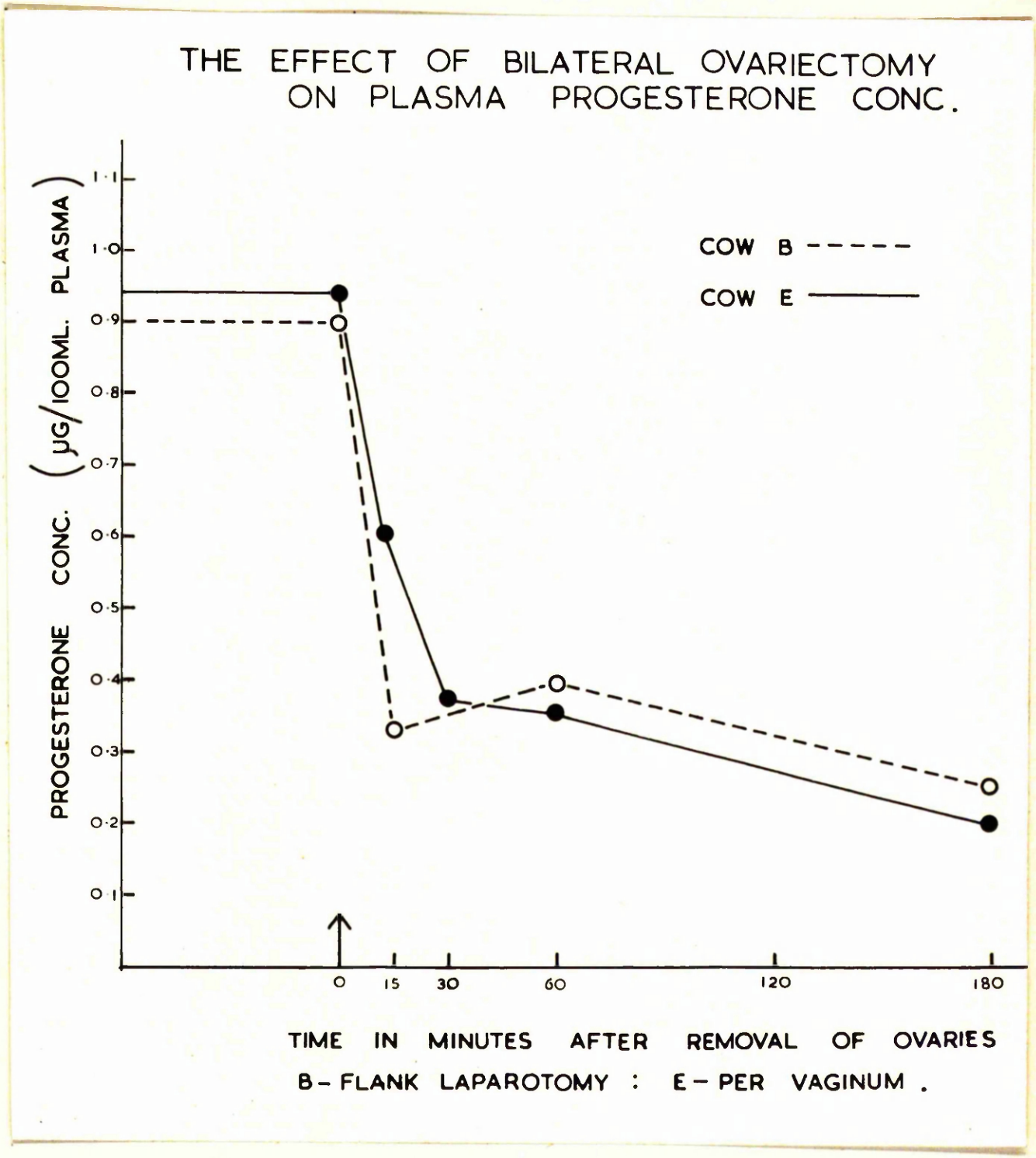
| Time in minutes after removing both ovaries | Volume of Plasma (ml) | Plasma Progesterone Concentration (µg/100 ml plasma) | Recovery of Radioactivity (%) |
|---|---|---|---|
| Control | 550 | 0.93 | 50 |
| 12 | 490 | 0.60 | 42 |
| 30 | 560 | 0.37 | 56 |
| 60 | 580 | 0.36 | 63 |
| 180 | 640 | 0.24 | 71 |
| 24 Hours | 620 | - | 42 |

Total weight of corpus luteum = 6.43 gms.

Progesterone content = 262.4 µg.

20B hydroxy progesterone = 32.5 µg.

FIGURE 3.B.12.


THE EFFECT OF BILATERAL OVARIECTOMY ON PLASMA PROGESTERONE CONC.
PROGESTERONE CONC. (µG/100ML. PLASMA)
1·1
1·0
0·9
0·8
0·7
0·6
0·5
0·4
0·3
0·2
0·1
COW B
COW E
0
15
30
60
120
180
TIME IN MINUTES AFTER REMOVAL OF OVARIES
B – FLANK LAPAROTOMY : E – PER VAGINUM .

## (3) PLASMA PROGESTERONE CONCENTRATION AFTER BILATERAL OVARIECTOMY IN THE COW AT MID CYCLE.

It was thought that the progesterone level remaining could have come from the residual ovarian tissue after enucleation of the corpus luteum. The same cows were bilaterally ovariectomised following the elapse of one normal cycle from the previous experiment. Cow B was ovariectomised via a flank laparotomy on day twelve and Cow E per vaginum on day fourteen. Again serial blood samples were taken immediately prior to and at known times after ovariectomy and the results for Cow B and E are shown in Tables 3.B.11. and 3.B.12. respectively. The changes in plasma progesterone concentration are also shown graphically in Figure 3.B.12. when it will be seen that the results do not differ from those obtained following surgical removal of the corpus luteum.

## DISCUSSION OF THE EFFECT OF REMOVAL OF THE CORPUS LUTEUM ON PLASMA PROGESTERONE CONCENTRATION.

The initial drop in progesterone level which occurs within the first 15 minutes following the removal of the corpus luteum is almost certainly due to the cessation of the main endogenous supply secretion and the known rapid disappearance of progesterone from the peripheral blood - in humans (Pearlman, 1957[b]; Zander, 1959; Short and Eton, 1959; and Sovia, Haskins and McCafferty, 1959) - in the rabbit (Zarrow, Shoger and Lazo-Wasem. 1954; and Rappoport, Goldstein and Haskins, 1957) - in the sheep (Bennet, Boursnell and Short, 1961 ; and Brush, 1961) - and in the cow (vide infra).

The levels represented by the secondary flat part of the curve could be due to a contribution from other sources of progesterone in the body, namely:

(a) Ovarian tissues: progesterone has been reported in ovarian tissues with all luteal tissue removed. (Gorski, et al. 1958 and Erb and Stormshak, 1961). In addition progesterone has been reported in ovarian vein blood from ovaries with only follicles or cysts present, in the sow

and ewe (Edgar, 1953[b]) and in the goat (Raeside and Turner, (1955).

(b) The adrenal glands: Balfour et al. have shown that bovine adrenal vein blood contains a considerable quantity of progesterone. Short (1960[a]) estimated a level of 1.5 mg/hr. in late pregnancy in the cow but pointed out that surgical stress might have contributed to the level.

(c) Body fat depots: Kaufmann and Zander (1956) have demonstrated the presence of progesterone in the body fat of women during the menstrual cycle, during pregnancy and following the injection of exogenous progesterone. In fact during pregnancy the level in the body fat appeared to be higher than that in the plasma. Davis, Plotz, Lupu and Ejarque (1960) using progesterone - 4 - C14, in pregnant women found the highest concentration of radioactivity in the body fat some hours after injection. As reported above in cows at the same stage of the cycle as the cows used in the present experiments, the progesterone levels in the body fat were 5 to 10 times higher than those in the plasma.

Following the elapse of one normal cycle, the effect of bilateral ovariectomy on plasma progesterone concentration, was essentially the same as that obtained

by removal of the corpus luteum. This indicates that a residual ovarian contribution is not responsible for the flat part of the curve. The fact that no progesterone could be detected 24 hours after surgery in any of the cows used in the above experiments suggests that a fat storage/release mechanism may be more important than an adrenal secretion. However, an adrenal contribution of progesterone lasting less than 24 hours due to post operative surgical stress cannot be ruled out completely.

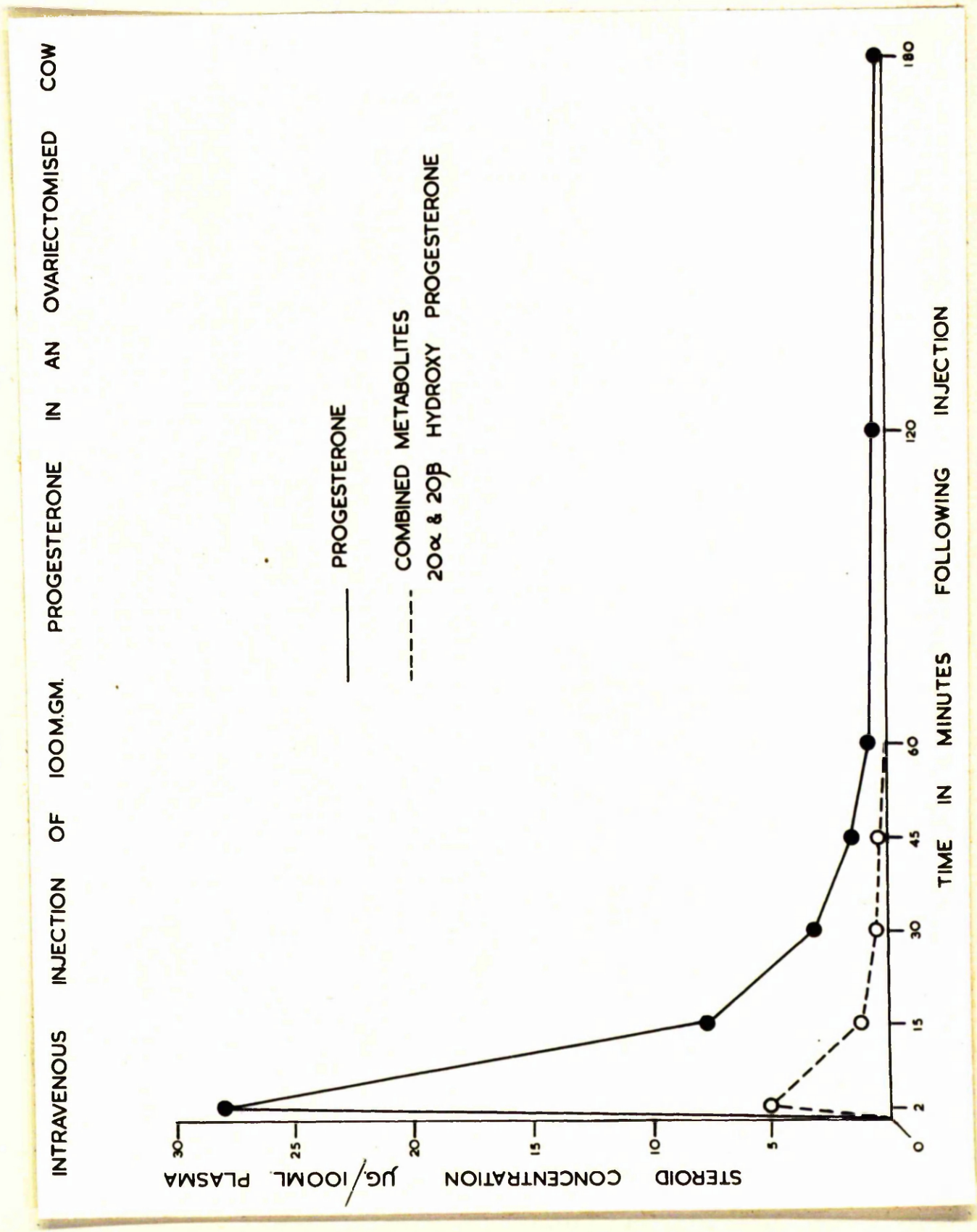
PLASMA CONCENTRATION OF PROGESTERONE AND ITS 20 - HYDROXY METABOLITES AFTER THE INTRAVENOUS INJECTION OF 100 mgm PROGESTERONE IN 10 ml ABSOLUTE ETHANOL.

OVARIECTOMISED COW (E)

| Time after injection in minutes | Volume of Plasma (ml) | Steroid concentration corrected for extraction losses (ug/100 ml plasma) | | | Recovery of Radioactivity (%) |
|---|---|---|---|---|---|
| | | Progesterone | 20α hydroxy-progesterone | 20β hydroxy-progesterone | |
| Control | 690 | - | - | - | 49 |
| 2 | 680 | 28.20 | 1.03 | 4.00 | 57 |
| 15 | 720 | 7.90 | 0.41 | 0.88 | 44 |
| 30 | 720 | 3.32 | 0.27 | 0.34 | 38 |
| 45 | 730 | 1.82 | 0.24 | 0.20 | 45 |
| 60 | 730 | 0.75 | - | - | 49 |
| 120 | 640 | 0.63 | - | - | 35 |
| 180 | 700 | 0.43 | - | - | 42 |

TABLE 3.B.13.

FIGURE 3.B.13.


INTRAVENOUS INJECTION OF 100M.GM. PROGESTERONE IN AN OVARIECTOMISED COW
PROGESTERONE
COMBINED METABOLITES
20α & 20β HYDROXY PROGESTERONE
STEROID CONCENTRATION µG/100ML PLASMA
TIME IN MINUTES FOLLOWING INJECTION
0
2
15
30
45
60
120
180
5
10
15
20
25
30

## (4.) METABOLISM OF INJECTED PROGESTERONE IN OVARIECTOMISED COWS.

The rapid rate at which progesterone disappears from the circulation has been reported for a number of animals (vide discussion) including man and in order to confirm that similar events occurred in the cow the following experiments were carried out:

100 mgm of Organon progesterone, previously tested chromatographically for chemical purity, was dissolved in 10 ml absolute ethanol and injected into the right mammary vein of an ovariectomised cow (F), from which a plasma blank had previously been withdrawn. Serial 1 litre blood samples were then collected by means of a wide bore jugular canula through which the flow was 1 litre of blood per minute. The results are expressed in Table 3.B.13. and shown graphically in Figure 3.B.13. where it will be seen that there is an extremely rapid removal of the exogenous progesterone from the blood.

In the two minute sample there were spots on the chromatogram which ran at identical Rf's to the two 20 hydroxy epimers of progesterone (20$\alpha$ and 20$\beta$) and whose combined

level was one fifth of that of the progesterone level indicating a rapid breakdown of the injected progesterone. From the initial slope of the curve it would appear that the half life (time for progesterone level to drop by half its original value) is of the order of 6 minutes which is comparable to that reported for other species. (Vide discussion).

It will be noted that although there was an appreciable level of the 20 hydroxy epimers at two minutes after injection, the level of these not only fell rapidly in parallel with progesterone, but, in addition, the ratio of the two epimers 20α to 20β altered.

In order to determine whether renal excretion was contributing to the rapid clearance of progesterone from the circulation, a similar experiment was performed in a normal cow. This animal had a specially designed "Ramshorn" urinary catheter in situ in the bladder through which continuous flow urine samples were collected. (Anderson and Pickering, 1961).

100 mgm. of progesterone in 10 ml ethanol was injected into the right jugular vein via a polythene catheter and serial 20 ml blood samples were withdrawn from the left jugular vein immediately prior to, and at known intervals after injection. At the same time 10 minute continuous flow

TABLE 3.B.14.

PLASMA PROGESTERONE CONCENTRATION AFTER THE INTRAVENOUS INJECTION OF 100 mgm PROGESTERONE IN ABSOLUTE ETHANOL - NORMAL COW.

| Time after injection in minutes | Volume of Plasma (ml) | Progesterone Concentration (µg/100 ml plasma) | Recovery of Radioactivity (%) |
|---|---|---|---|
| Control | 18 | nil | 62 |
| 0.5 | 13 | 52.4 | 68 |
| 1.0 | 12 | 89.6 | 48 |
| 2.5 | 12 | 39.2 | 43 |
| 10.5 | 51 | 15.1 | 56 |
| 15.0 | 105 | 9.4 | 61 |

urine samples were collected via the urinary catheter immediately pre and post injection. The results are presented in Table 3.B.14. where it will be seen that the rate of progesterone clearance from the blood is similar to that obtained previously. In this case it was not possible to detect either of the 20 epimers of progesterone as only volumes of blood large enough to measure progesterone satisfactorily were withdrawn.

A small amount of progesterone (2.6 µg) was found in the second urine sample (300 ml) which represents an extremely small fraction of the total amount injected and thus renal clearance of progesterone per se does not appear to be of significance.

In this experiment the jugular vein blood samples were withdrawn more frequently starting from 0.5 minutes following injection. The results indicate that progesterone is detectable as early as 0.5 minutes and that a peak level is reached at about one minute after injection. An extremely rapid disappearance of progesterone from the plasma then follows as described previously.

In order to confirm further the identity of the 20 epimers another ovariectomised cow was injected intra-

venously with 100 mgm of progesterone and two litres of blood were withdrawn within 5 minutes of injection. This sample yielded both 20 epimers in addition to progesterone and the ratio of the three compounds was similar to that seen in the two minute samples in the first ovariectomised cow used. The two epimers were tentatively identified by their Rf values on paper chromatograms, by their absorption spectra in ethanol, and by the formation of acetates (Zander et al.1958; Bush, 1961).

## DISCUSSION OF METABOLISM OF INJECTED PROGESTERONE IN THE COW.

The rapid removal of injected progesterone from the peripheral blood has been reported for a number of different species. The possible routes by which this could occur are by diffusion into the body tissues including uptake by protein binding, catabolism of the progesterone molecule or by renal exretion. The results of this study indicate that even with a massive dose catabolism of the injected progesterone to both 20$\alpha$ and 20$\beta$-hydroxy-progesterone takes place within two minutes of injection; these metabolites amounted to about one fifth of the progesterone circulating at that time.

The presence of the 20$\alpha$ epimer is interesting as only the 20$\beta$ epimer has been demonstrated in adult bovine tissues. The exception being the presence of the 20$\alpha$ epimer in the adrenal vein blood of the calf prior to the 64th day of life (Dalfour et al. 1959). Short (1958[b]) demonstrated a slow conversion of progesterone by whole bullock blood to the 20$\beta$ epimer when incubated at 37°. Hayano et al. (1954) demonstrated the <u>in vitro</u>

conversion of progesterone to the 20β epimer by bovine luteal tissue homogenates with added progesterone.

The main site of progesterone catabolism is in the liver, as in hepatectomised rabbits the rate of removal from the circulation is markedly reduced compared to controls, (Haskins, 1950). In the hepatectomised rats a slow conversion to the 20 epimer takes place in extra-hepatic tissues (Berliner and Wiest, 1956; and Wiest, 1956).

A possible explanation for the appearance of the 20α epimer in this instance is that the 20β hydroxylase enzyme equilibrium became upset and therefore more substrate was available for 20α hydroxylation.

There is a considerable species variation as to which epimer is formed. In the human subject the 20α epimer predominates in the placenta, luteal tissue and in blood (Short, 1957; Zander et al. 1958, and Short, 1960[c]). This is in keeping with the presence of pregnanediol (5β-pregnane-3α:20αdiol) as the principal metabolite in pregnant women's urine.

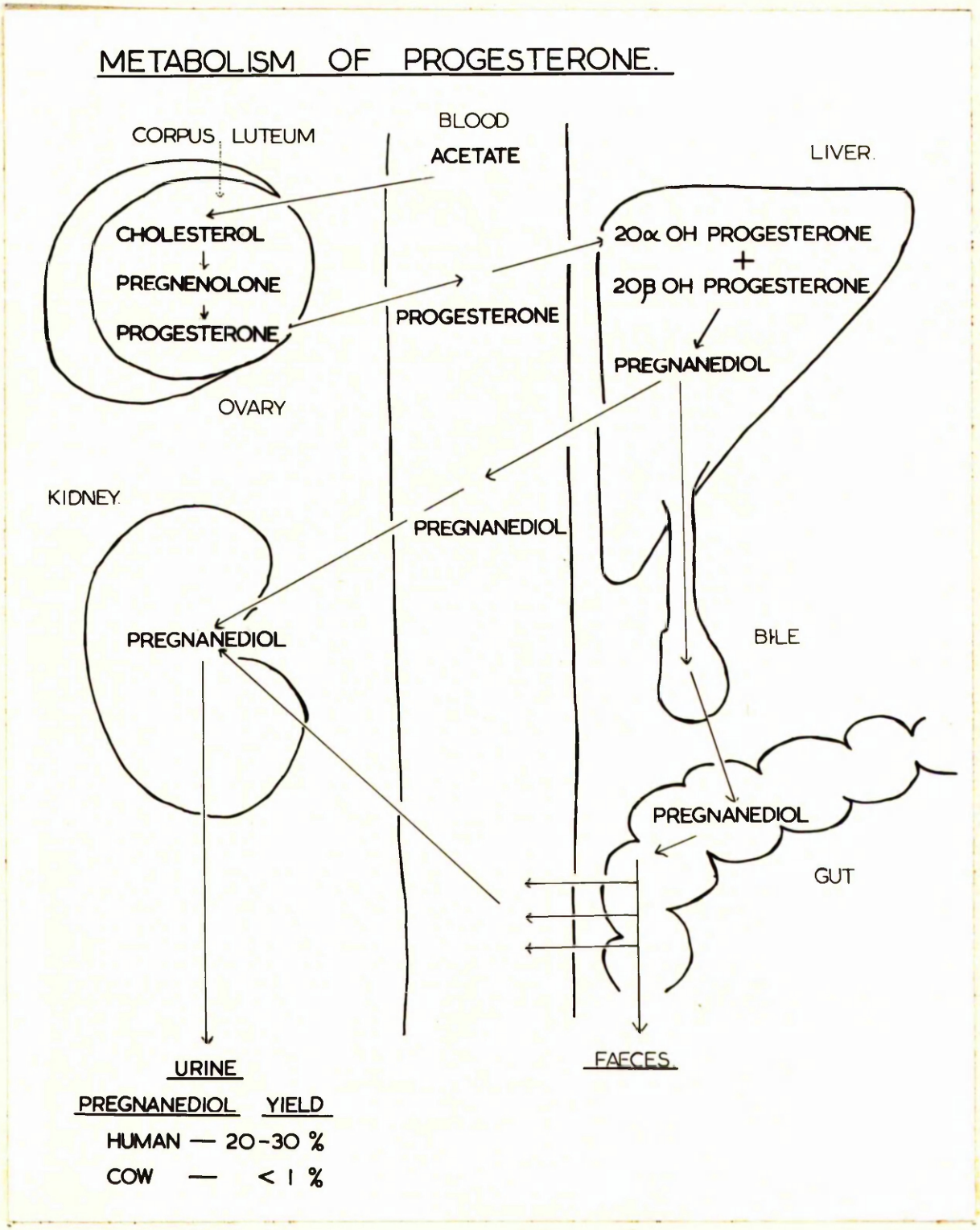
When progesterone is incubated with human liver *in vitro*, reduction of the 20 ketone groups is always

accompanied by reduction of the A-ring as well (Atherden, 1959). It may be that with massive doses of progesterone the enzyme responsible for A-ring reduction in the bovine is more readily overloaded than that responsible for the reduction of the 20 ketone group thus allowing an accumulation of the 20 epimers. Alternatively the formation of the 20 epimers may be a result of extra hepatic metabolism as in addition to the work of Berliner et al. (1956) it has been shown that the 20 epimers are formed in tissue cultures of human uterine fibroblasts (Sweat, Grosser, Berliner, Swim, Nabors and Dougherty, 1958) and also in human placental tissue (Little, DiMartinis and Nyholm, 1959).

The fact that 5β-pregnane-3∝ :20∝ diol is present in only trace amounts in cow's urine (Klyne and Wright, 1959) and that Pearlman and Cerceo (1948), in a study of pregnant cow bile, found large amounts of 5β-pregnane-3∝ :20β diol, suggests that the 20β hydroxylation of progesterone under normal conditions is the predominant pathway.

The rapid removal of progesterone from peripheral blood has already been discussed. (vide supra). From Figure 3.B.13. it will be seen that the half life of progesterone (time taken for the blood level to fall by half)

**FIGURE 3.B.14.**


METABOLISM OF PROGESTERONE.
CORPUS LUTEUM
BLOOD
ACETATE
LIVER.
CHOLESTEROL
PREGNENOLONE
PROGESTERONE
OVARY
PROGESTERONE
20α OH PROGESTERONE
+
20β OH PROGESTERONE
PREGNANEDIOL
KIDNEY.
PREGNANEDIOL
PREGNANEDIOL
BILE
PREGNANEDIOL
GUT
URINE
FAECES
PREGNANEDIOL YIELD
HUMAN — 20-30 %
COW — < 1 %

is in the order of 6 minutes which compares with an equivalent of 5 minutes for women (Pearlman, 1957; Short and Eton, 1959). The sheep on the other hand has a slightly shorter half life at 4 minutes (Bennet, Boursnell and Short, 1961).

The removal of "free" progesterone from the circulation due to binding with the plasma protein is not very likely in view of the recent finding of Forbes, Coulombre and Coulombre (1961) who studied the inactivation of progesterone by rat liver in vitro and found that protein binding did not occur to a significant extent.

The pathways of progesterone metabolism and the comparative yields of urinary pregnanediol in pregnant women and in the cow are shown diagramatically in Figure 3.B.14.

TABLE 3.B.15.

PROGESTERONE IN THE BODY FAT OF COWS.

| Days since oestrus | Amount of fat (gms) | Progesterone | | |
|---|---|---|---|---|
| | | Fat * µg/100 gms | Plasma µg/100 ml | Corpus luteum ug/gland |
| 13 | 400 | 6.4 | 0.92 | 147 |
| 13 | 960 | 5.8 | 1.21 | 182 |
| 1 | 850 | < 1 µg | 0.11 | 23 |
| ovariectomised cow. | 420 | < 1 µg | nil | nil |
| bullock. | 380 | < 1 µg | nil | nil |

* Not corrected for extraction losses.

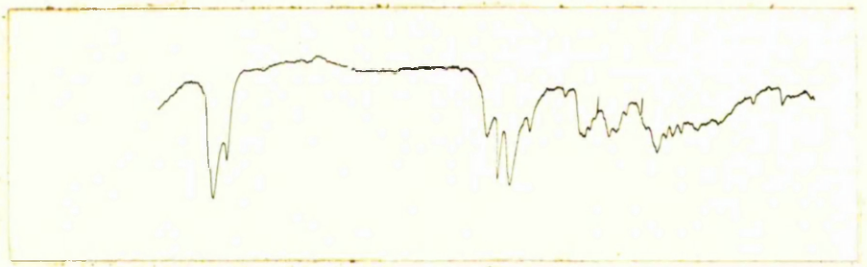
## (5). PROGESTERONE IN THE BODY FAT OF COWS.

Following the observation reported above that progesterone is so rapidly removed from the circulation in the cow and that a residual level persisted for some hours after injection, it was thought that the body fat might act as a reservoir for progesterone. Accordingly it was decided to examine the body fat of normal cows for the presence of progesterone and/or its metabolites. Body fat, blood and luteal samples were obtained at slaughter from a small number of cows which had not previously been treated with progesterone and which were at a known stage of the oestrous cycle. The fat, which was obtained from the kidney region, was extracted as described in the Methods Section and the results for the various tissues are shown in Table 3.B.15. It will be seen that at mid cycle (13 days) the level of progesterone in body fat is between 5 and 10 times the level of that found in the plasma (assuming that 100 gm of fat is equivalent to 100 ml plasma) while at oestrus, in ovariectomised cows and in bullock fat the level was comparatively low.

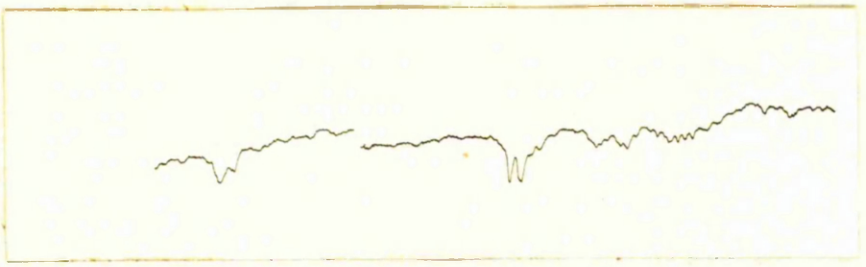
The progesterone obtained from the body fat was tentatively identified by its Rf values in Bush system A,

## FIGURE 3.B.15.

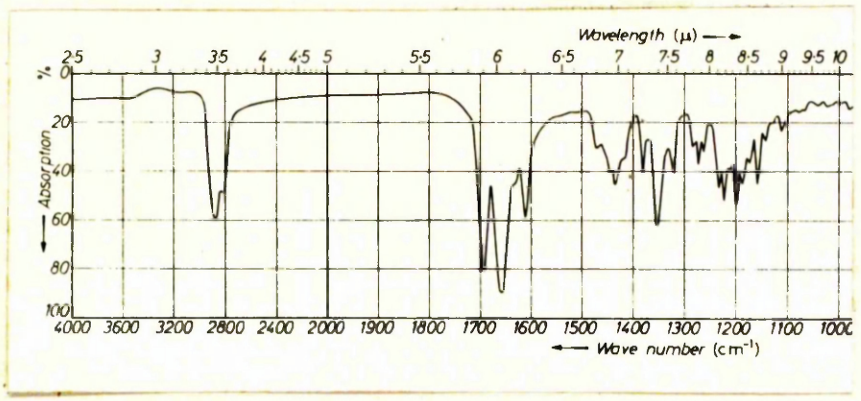
## INFRA-RED SPECTRUM OF PROGESTERONE ISOLATED FROM BODY FAT (A)



A.



B.




C.

by its ultra-violet absorption spectrum in ethanol and by the blue colour which developed on application of the Zimmerman reagent. In addition the compound gave a yellow sodium hydroxide fluorescence as described by Bush (1952).

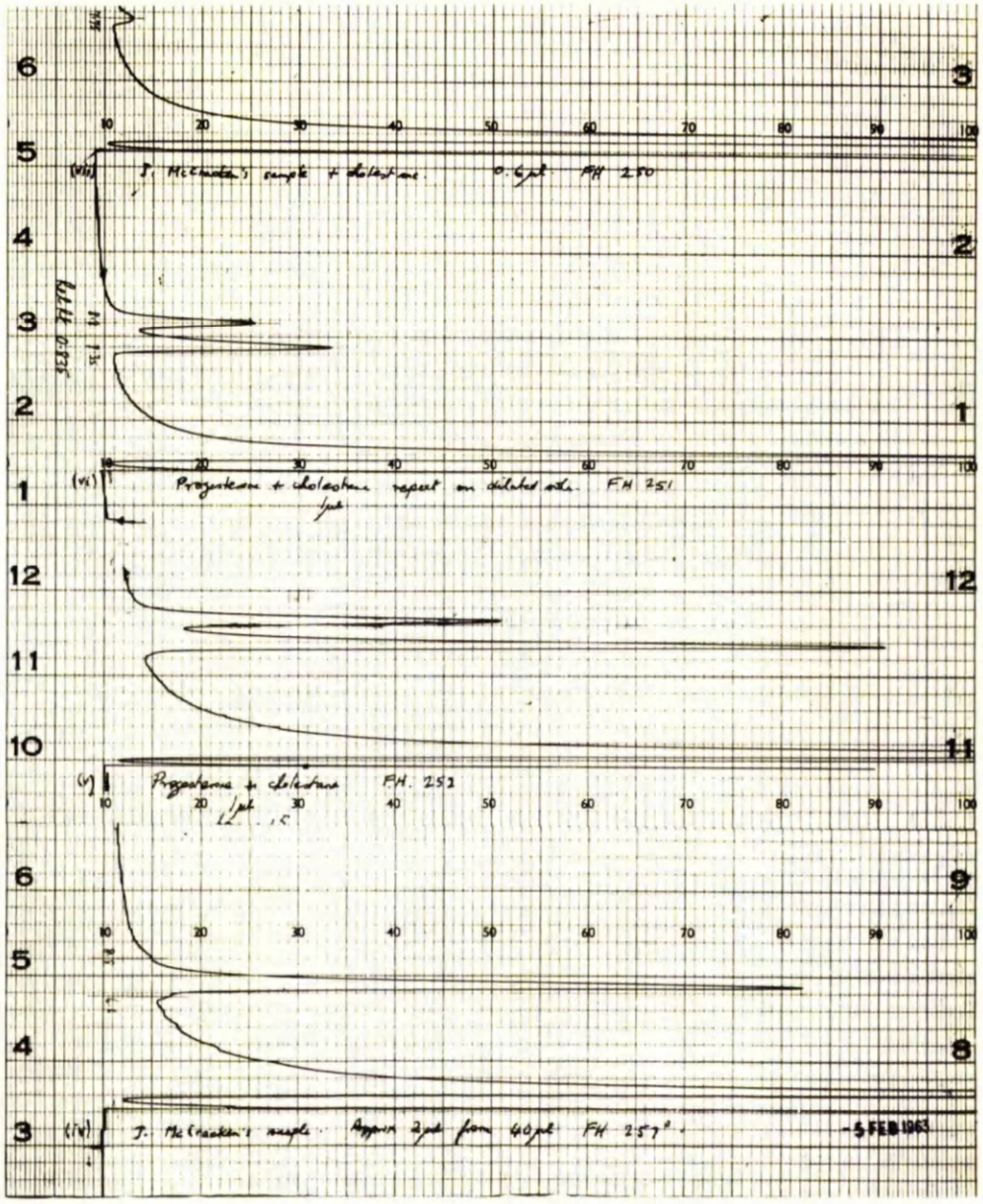
For further identification the steroid compound (approximately 80 µg) obtained from the body fat of the two cows at mid cycle was subjected to infra red analysis. Figure 3.B.15. shows the spectrum of the unknown compound (A) as compared with 50 µg "Organon" progesterone (B) and the infra red reference spectrum of authentic progesterone (C). This analysis confirmed the presence of progesterone and revealed the presence of an additional small peak in the "red region" which was tentatively identified as an acetate grouping.

In order to further confirm the presence of progesterone and if possible to identify this trace of impurity a small part of the sample was subjected to gas/liquid chromatography. In this analysis the retention time of the sample relative to cholestane was identical to that of authentic progesterone at 0.84. Furthermore, from the shape of the peak obtained with the sample it was

FIGURE 3.B.16.

GAS/LIQUID CHROMATOGRAM OF PROGESTERONE

ISOLATED FROM BODY FAT.



apparent that it represented a sample of at least 95% pure progesterone. (Figure 3.B. 16.). The balance could be accounted for by trace amounts of either other steroids or their acetates. This possible presence of acetate is not surprising since acetic anhydride was used in the final purification of the sample.

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Reference - unpublished at binding

"The correlation of gas/liquid chromatographic behaviour and structure of steroids."

C.J.W. BROOKS, and L. HANAINEH. Biochem. J. (1963) 87, 151.

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## DISCUSSION

Kaufman and Zander (1956) reported the presence of progesterone in the body fat of women during pregnancy, during the menstrual cycle and after injection of exogenous progesterone. The level they found in fat in late pregnancy was 170 µg/100 gm fat tissue, which is considerably higher than the levels found in plasma at this time - 5 to 35 µg/100 ml plasma. (Zander, 1954 and 1955; Sommerville, 1957 and Eton and Short, 1959). In addition, Plotz and Davis (1957) using progesterone - 4 - C14 found the highest concentration of radioactivity in the body fat for some hours after injection.

The presence of progesterone in the body fat of cows, at a level higher than that in the plasma, is therefore not surprising. It is interesting to speculate as to the role of the body fat as a depot for progesterone and possibly other steroid hormones. It is probable that diffusion into the body fat will partly account for the rapid disappearance of injected progesterone in the cow as is the case in the rabbit (Zarrow et al. 1954). The residual level of 0.5 µg/100 ml which persisted in the ovariectomised cows for some hours after the injection of

progesterone might indicate a slow release of this substance from the fat depots back into the circulation. The same reason would explain the plasma level of progesterone persisting after removal of the ovaries in the experiments described above.

If the body fat does act as a reservoir of progesterone it might prevent gross fluctuation in the blood level if the endogenous supply was to alter markedly. Assuming that the amount of fat tissue in a cow weighing 500 kg. is 75 kg. (Morrison, 1959) and that the concentration of progesterone is 10 µg/100 gms then the total amount of progesterone stored would be 7.5 mg. This amount, provided it were released at a suitable rate, could probably maintain a blood level for some hours (Short,1959).

The comparatively low levels found in the fat of the cow at oestrus, in the ovariectomised cow and in the steer would indicate a low endogenous production of progesterone in these animals. The only fat samples in which progesterone - 4 - C14 was utilised as an internal marker were the ones from the ovariectomised cow and the steer in which the recoveries were 37 and 24% respectively. This is not surprising in view of the number of purifications

necessary with large quantities of fat tissue. Progesterone - 4 - C14 was not used during the extraction with the fat from the cows at mid-cycle as it was desirable that the progesterone should be uncontaminated as further identification of the progesterone by physical means was anticipated.

## (6) PROGESTERONE CONCENTRATION IN MILK.

In view of the fact that progesterone was found to be widely distributed in the body tissues, litre samples of milk were collected from two cows and examined for progesterone content. These were processed in exactly the same way as plasma samples except that, owing to the high fat content of the milk (approximately 4%), the residue had to be de-fatted by the freezing aqueous methanol procedure adopted by Allen (1932). Milk obtained from a cow 92 days pregnant contained 8.6 µg of progesterone per litre. On the other hand milk obtained from a cow three days post oestrus did not contain any detectable progesterone. The recovery of radio-progesterone added to these two milk samples was 37% and 24% for the pregnant and non-pregnant cows respectively.

## DISCUSSION

From the above results it is apparent that the concentration of progesterone in milk is approximately the same as that found in plasma. The concentration in the pregnant cow's milk was 0.86 µg/100 ml which is similar

to the plasma level found at this stage in pregnancy by Short (1958[b]). Similarly the failure to detect progesterone in the milk sample obtained from the cow early in the cycle agrees with the low level found in plasma at this time in the present study. Had the recovery from this sample been higher it might have been possible to detect some progesterone in this sample.

In view of the high fat content of milk and the difficulty in removing it during processing, there would appear to be no advantage of this material over plasma for the investigation of progesterone levels in the cow.

## SECTION 3 - RESULTS

### C. PROGESTERONE STATUS OF THE COW DURING EARLY PREGNANCY.

(1) Progesterone concentration in peripheral blood and in the corpus luteum in cows 16 days after insemination - pregnant and non-pregnant.

(2) Progesterone concentration in peripheral blood and in the corpus luteum in cows 26 days after insemination - pregnant and non-pregnant.

(3) Progesterone level in peripheral blood in selected cases two days prior to slaughter.

(4) Relationship between progesterone concentration in blood and total corpus luteum contents at 16 and 26 days after insemination.

(5) Relationship between the progesterone content of the corpus luteum and the gland weight at 16 and 26 days after insemination.

(6) 20β levels in the corpus luteum.

## SECTION 3 - RESULTS

### C. PROGESTERONE STATUS OF COWS IN EARLY PREGNANCY. (1. & 2.)

Introduction: A unique opportunity arose to examine the progesterone status of cows in early pregnancy during an investigation at the Veterinary Hospital into early embryonic death in cattle. This study was undertaken by Drs. Boyd, Bacsich and Young with the financial support of the Agricultural Research Council.

Plan of Experiment: An integral part of this study was that the investigation into embryonic losses in normal cows should be carried out under farm conditions. Accordingly, a group of 40 cows were selected individually over a period of one year from neighbouring farms. The breeding history and physical state of each animal, particularly its genitalia, were used to assess normality. When an animal was selected it was artificially inseminated on the farm within 24 hours of oestrus having been observed by the farmer and brought into the Veterinary Hospital for slaughter at a known time after insemination. One half of the cows were slaughtered at 16 days and the other half at 26 days after insemination.

TABLE 3.C.1.

PROGESTERONE STATUS IN COWS - 16 DAYS NON-PREGNANT.

| Cow Number | Plasma Progesterone Concentration (µg/100 ml plasma) | Progesterone in Corpus Luteum (Total content in µg) | Total Weight of Corpus Luteum (Weight in grams) |
|---|---|---|---|
| 1 | 0.85 | 255 | 6.20 |
| 12 | 0.91 | 258 | 6.39 |
| 14 | 1.08 | 332 | 5.71 |
| 20 | 1.24 | 427 | 7.33 |
| 23 | 0.95 | 258 | 4.63 |
| 30 | 0.91 | 197 | 5.53 |
| 32 | 0.65 | 253 | 6.28 |
| 33 | 0.83 | 298 | 5.40 |
| Mean = | 0.93 | 284.7 | 5.93 |
| S.D. = | 0.18 | 69.0 | 0.6 |
| S.E. = | 0.023 | 8.7 | 0.08 |

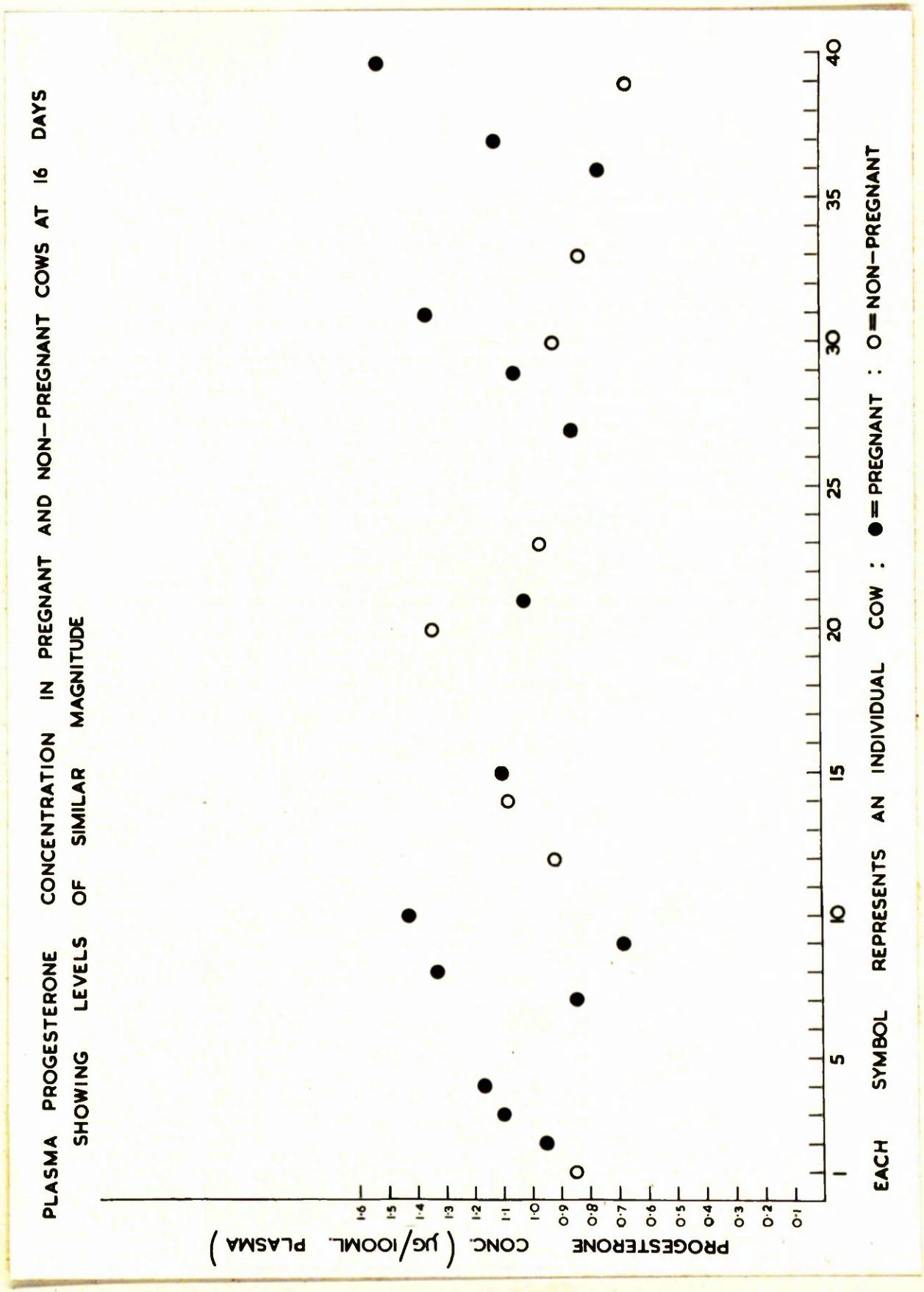
TABLE 3.C.2.

PROGESTERONE STATUS IN COWS - 16 DAYS PREGNANT

| Cow Number | Plasma Progesterone Concentration (µg/100 ml plasma) | Progesterone in Corpus Luteum (Total content in µg) | Total Weight of Corpus Luteum (Weight in grams) |
|---|---|---|---|
| 2 | 0.96 | 253 | 6.07 |
| 3 | 1.10 | 337 | 7.53 |
| 4 | 1.18 | 324 | 5.47 |
| 7 | 0.83 | 236 | 4.95 |
| 8 | 1.34 | 309 | 5.91 |
| 9 | 0.68 | 213 | 4.52 |
| 10 | 1.44 | 335 | 7.85 |
| 15 | 1.10 | 263 | 5.39 |
| 21 | 0.91 | 300 | 6.08 |
| 29 | 1.05 | 220 | 4.90 |
| 31 | 1.47 | 336 | 6.19 |
| 36 | 0.76 | 333 | 6.67 |
| 37 | 1.12 | 353 | 7.21 |
| 40* | 1.51 | 365 (229 / 136) | 7.30 (3.70 / 3.60) |
| Mean = | 1.10 | 298 | 6.15 |
| S.D. = | 0.26 | 51 | 1.05 |
| S.E. = | 0.018 | 3.67 | 0.057 |

* Twin Corpora Lutea

FIGURE 3.C.1.


PLASMA PROGESTERONE CONCENTRATION IN PREGNANT AND NON-PREGNANT COWS AT 16 DAYS
SHOWING LEVELS OF SIMILAR MAGNITUDE
EACH SYMBOL REPRESENTS AN INDIVIDUAL COW : ●= PREGNANT : O= NON-PREGNANT
PROGESTERONE CONC. (µG/100ML. PLASMA)
1·6
1·5
1·4
1·3
1·2
1·1
1·0
0·9
0·8
0·7
0·6
0·5
0·4
0·3
0·2
0·1
1
5
10
15
20
25
30
35
40

Collection of Samples: One litre blood samples were obtained by jugular puncture one hour prior to slaughter. To provide additional information blood samples were collected on the farm from certain animals two days prior to slaughter, i.e. at 14 and 24 days in the 16 and 26 day groups, respectively. At slaughter the ovaries were removed as quickly as possible and the corpus luteum or corpora lutea were dissected out. Each gland was weighed and a representative sample weighing approximately 0.5 gm removed for progesterone estimation.

Results: The procedure for progesterone estimation was carried out as before on the plasma samples and corpora lutea obtained from these animals. In addition, with corpus luteum samples it was possible to measure the amounts of 20β hydroxy progesterone present in the gland. The results in the 16 day group of cows pregnant and non-pregnant are shown in Table 3.C.1. and 3.C.2. respectively. It will be seen that the levels in both the plasma and corpora lutea of the two groups do not differ markedly. A visual comparison of the plasma concentration between cows pregnant and non-pregnant at 16 days can be seen in Figure 3.C.1. Here, each symbol represents the level of

TABLE 3.C.3.

PROGESTERONE STATUS IN COWS - 26 DAYS PREGNANT

| Cow Number | Plasma Progesterone Concentration (µg/100 ml plasma) | Progesterone in Corpus Luteum (Total content in µg) | Total Weight of Corpus Luteum (Weight in grams) |
|---|---|---|---|
| 6 | 1.60 | 331 | 5.78 |
| 11 | 1.20 | 337 | 7.40 |
| 13 | 0.92 | 239 | 6.34 |
| 16 | 1.35 | 421 | 7.01 |
| 17 | 1.63 | 454 | 8.60 |
| 18 | 0.88 | 301 | 4.56 |
| 19 | 1.10 | 257 | 5.80 |
| 22 | 1.28 | 322 | 6.43 |
| 24 | 0.86 | 192 | 3.75 |
| 26* | 1.41 | 414 (194, 220) | 7.89 (4.22, 3.67) |
| 27 | 0.85 | 211 | 5.41 |
| 35 | 0.91 | 257 | 5.44 |
| 41 | 0.89 | 268 | 6.50 |
| Mean = | 1.14 | 308 | 6.22 |
| S.D. = | 0.29 | 81 | 1.33 |
| S.E. = | 0.08 | 22 | 0.10 |

* Twin Corpora Lutea.

TABLE 3.C.4.

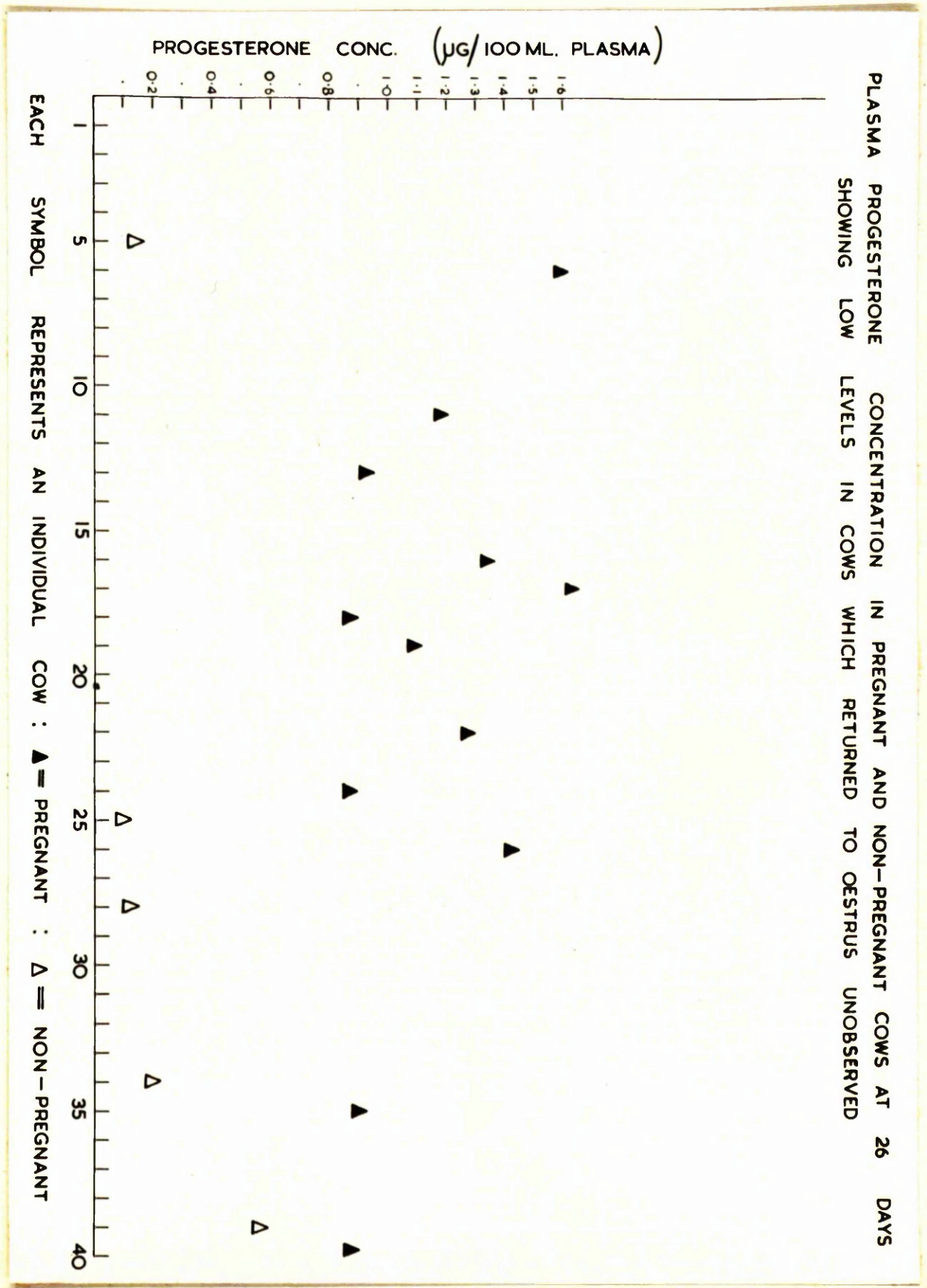
PROGESTERONE STATUS IN COWS - 26 DAYS NON-PREGNANT

(The ovaries of each animal contained both a regressing and a developing corpus luteum). *

| Case Number | Plasma Progesterone Concentration (µg/100 ml plasma) | Progesterone in corpus luteum (µg/gland) | | Total Weight of Corpus Luteum (in gms) | |
|---|---|---|---|---|---|
| | | Old | New | Old | New |
| 5* | 0.12 | 10.3 | - | 0.23 | - |
| 25 | 0.11 | 18.8 | 10.6 | 0.90 | 0.46 |
| 28 | 0.12 | 2.1 | 24.1 | 0.52 | 0.66 |
| 34 | 0.22 | - | 40.8 | 0.28 | 1.67 |
| 39 | 0.51 | - | 124.8 | 0.14 | 5.20 |

* Cow No.5 which was subsequently withdrawn from the series due to the presence of cystic ovarian degeneration had only one regressing corpus luteum.

FIGURE 3.C.2.




PLASMA PROGESTERONE CONCENTRATION IN PREGNANT AND NON-PREGNANT COWS AT 26 DAYS SHOWING LOW LEVELS IN COWS WHICH RETURNED TO OESTRUS UNOBSERVED

EACH SYMBOL REPRESENTS AN INDIVIDUAL COW : ▲ = PREGNANT : Δ = NON-PREGNANT

progesterone of an individual animal at the time of slaughter. The ordinate of the graph shows the animals numbered 1 to 40, in the sequence in which they were inseminated and slaughtered. Only half the animals were of course slaughtered at 16 days. The selection of animals for slaughter at either 16 days or 26 days after insemination was purely at random.

The results in the 26 day group of cows, pregnant and non-pregnant, are presented in Tables 3.C.3 and 3.C.4. respectively, and in addition shown graphically in Figure 3.C.2. It can be seen that there is a marked difference in progesterone concentration in both plasma and in corpora lutea between the two groups. This is in agreement with the finding that the ovaries of the non-pregnant cows contained a new growing corpus luteum and an old regressing one except for cow No.5 which had only a small regressing corpus luteum and no signs of a recent ovulation. This animal was subsequently deleted from the series due to evidence of cystic ovarian degeneration.

From the ovarian findings at slaughter it would appear that all the cows non-pregnant at 26 days had recently ovulated. Had any of these animals been detected

in oestrus they would have been re-inseminated. The fact that these animals were not reported in oestrus indicates that either they did not manifest clinical signs (silent heat) or that the farmer either failed to observe oestrus or failed to report the occurrence of oestrus.

Of the cows initially designated to the 26 day group, three animals were detected in oestrus by the farmer prior to slaughter at 26 days. The animals in question were cows No. 7, 12 and 36 which had returned to oestrus by days 16, 22 and 19, respectively.

Nos. 7 and 12 were re-allocated to the 16 day group and number 36 was re-allocated to the 26 day group but was again detected in oestrus prior to slaughter, this time on day 20. On this occasion the animal was re-allocated to the 16 day group and when slaughtered 16 days post insemination it was found to be pregnant.

Cow No.39 shows a higher concentration of progesterone in the plasma and in a corpus luteum of a greater weight than the others in the group. This is, however, compatible with a return of oestrus between 18 and 20 days after insemination, and the formation of a new corpus luteum

which was 6 to 8 days old at slaughter. This in turn would produce a plasma progesterone concentration of approximately 0.5 µg/100 ml plasma as seen in cows during days 6 to 8 of the oestrous cycle, and in this subject cow No.39.

TABLE 3.C.5.

PLASMA PROGESTERONE CONCENTRATION AT 14 & 16 DAYS AFTER INSEMINATION.

(µg/100 ml plasma)

| PREGNANT | | | NON-PREGNANT | | |
|---|---|---|---|---|---|
| Cow No. | 14 Days | 16 Days | Cow No. | 14 Days | 16 Days |
| 29 | 1.16 | 1.05 | 23 | 0.84 | 0.95 |
| 31 | 1.64 | 1.47 | 30 | 0.98 | 0.91 |
| 37 | 1.35 | 1.12 | 33 | 0.80 | 0.83 |
| Means | 1.38 | 1.21 | | 0.87 | 0.90 |

TABLE 3.0.6.

PLASMA PROGESTERONE CONCENTRATION AT 24 & 26 DAYS AFTER INSEMINATION.

(µg/100 ml plasma)

| PREGNANT | | | NON PREGNANT | | |
|---|---|---|---|---|---|
| Cow No. | 24 Days | 26 Days | Cow No. | 24 Days | 26 Days |
| 11 | 1.12 | 1.20 | 25 | 0.12 | 0.11 |
| 16 | 1.23 | 1.35 | 28 | 0.12 | 0.12 |
| 22 | 1.19 | 1.28 | 34 | 0.10 | 0.22 |
| 24 | 0.88 | 0.86 | 39 | 0.35 | 0.51 |
| 26* | 1.35 | 1.41 | | | |
| 35 | 0.85 | 0.91 | | | |
| Mean: | 1.10 | 1.17 | | 0.17 | 0.24 |

* Twin corpora lutea.

3. PLASMA PROGESTERONE CONCENTRATION IN THE 16 AND 26 DAY

3. PLASMA PROGESTERONE CONCENTRATION IN THE 16 AND 26 DAY

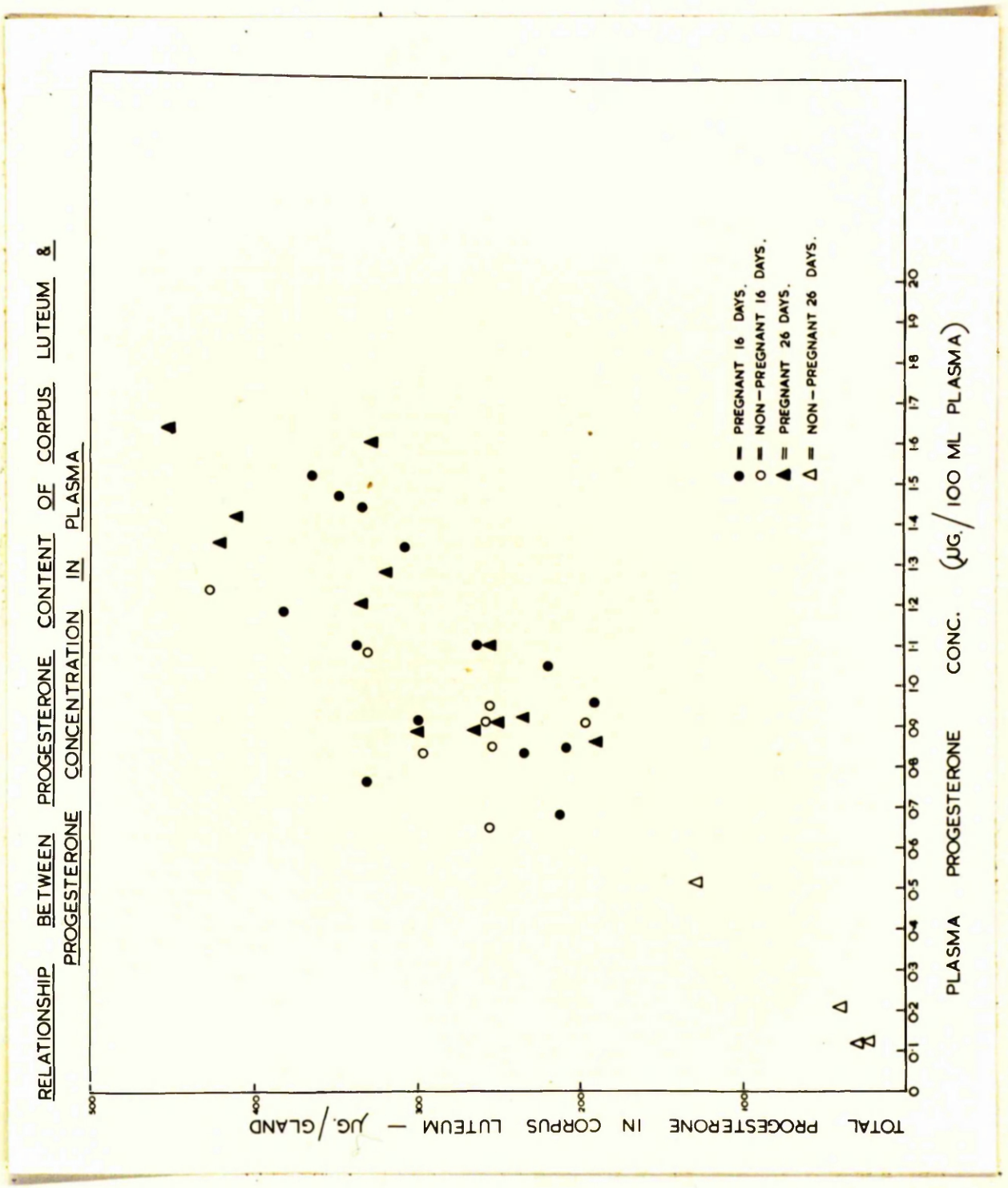
GROUPS TWO DAYS PRIOR TO SLAUGHTER.

To provide additional information blood samples were collected, whenever possible, on the farm two days prior to the cows being slaughtered at the Veterinary Hospital.

The results obtained in the 16 day group are shown in Table 3.C.5. where it will be seen that in both the pregnant and non-pregnant animals the levels on day 14 and 16 are of a similar order. It will be seen that in this rather small sample of animals the mean level of the non-pregnant cows is somewhat lower than the mean level of the pregnant cows.

Table 3.C.6. shows the results obtained in the 26 day group. It will be seen that there is a similarity between the 24 and 26 day levels of the individual cow in both the pregnant and non-pregnant animals but that there is a marked difference between the pregnant and non-pregnant animals as a whole.

FIGURE 3.C.3.


RELATIONSHIP BETWEEN PROGESTERONE CONTENT OF CORPUS LUTEUM &
PROGESTERONE CONCENTRATION IN PLASMA
TOTAL PROGESTERONE IN CORPUS LUTEUM — µG./GLAND
PLASMA PROGESTERONE CONC. (µG./100 ML PLASMA)
● = PREGNANT 16 DAYS.
○ = NON-PREGNANT 16 DAYS.
▲ = PREGNANT 26 DAYS.
Δ = NON-PREGNANT 26 DAYS.

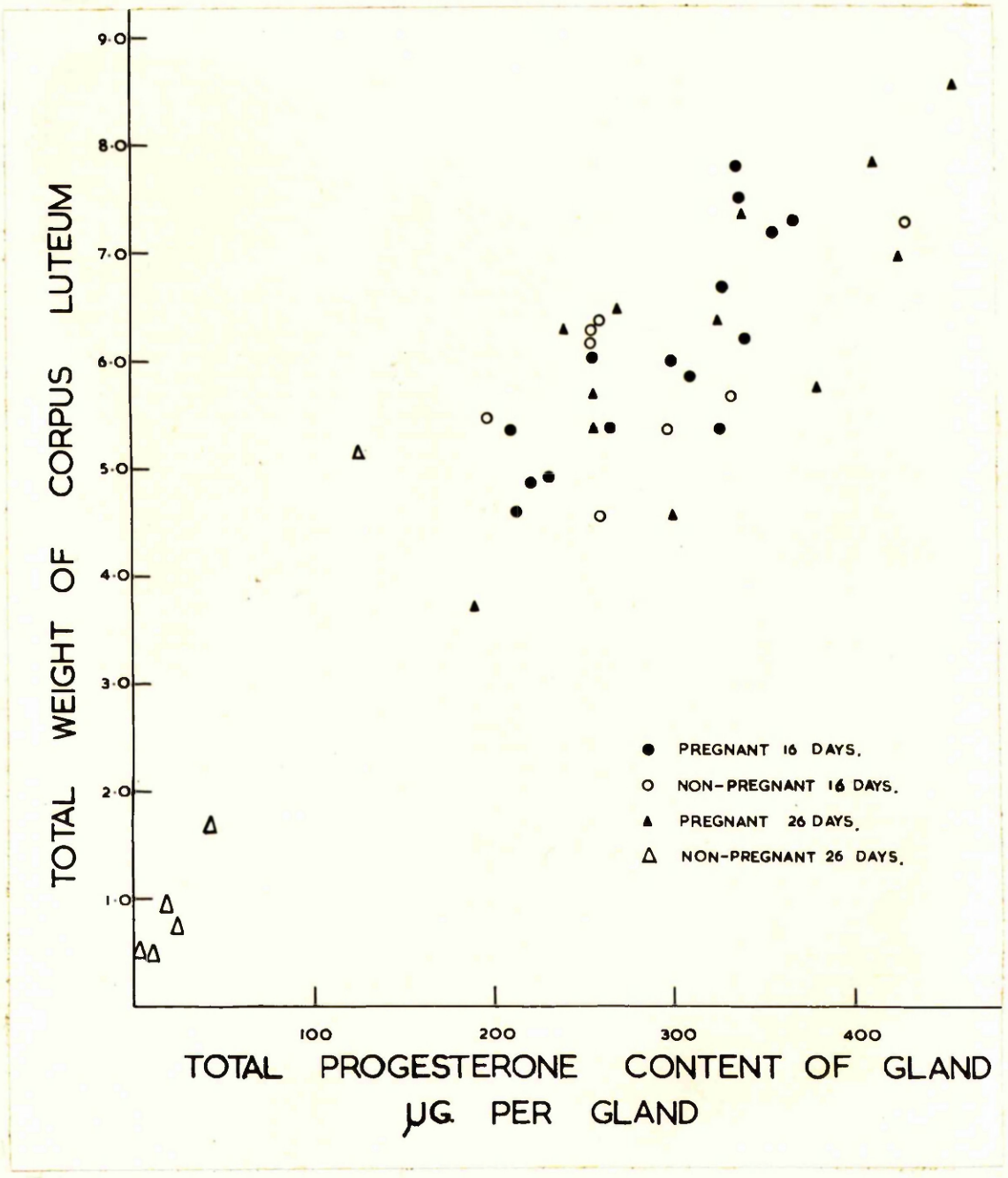
## 4. RELATIONSHIP BETWEEN THE PROGESTERONE CONTENT OF THE CORPUS LUTEUM AND THE PROGESTERONE CONCENTRATION IN PLASMA.

Using the results obtained in the 16 and 26 day groups it was possible to construct a graph to show the relationship in each cow between the total amount of progesterone found in the corpus luteum and the progesterone concentration present in the plasma.

It will be seen in Figure 3.C.3. that the glands containing the greatest amount of progesterone appear to be associated with the highest levels of progesterone found in the plasma. Conversely the glands containing the lowest quantities appear to be associated with low plasma progesterone levels. The relationship is on the whole not well defined for it will be seen that at a given level of progesterone in the gland the plasma concentration often shows a range of readings differing by as much as 0.5 ug. which may represent a difference of 50% between readings.

FIGURE 3.C.4.

RELATION BETWEEN GLAND WEIGHT AND PROGESTERONE CONTENT.



## 5. RELATIONSHIP BETWEEN THE TOTAL PROGESTERONE CONTENT AND THE TOTAL WEIGHT OF THE CORPUS LUTEUM.

In order to demonstrate the relationship between the corpus luteum weight and its progesterone content results from the 16 and 26 day groups were used to construct a graph where the total content of progesterone in the corpus luteum was plotted against the weight of the gland for each cow in the series.

Figure 3.C.4. shows the results obtained and it will be seen that progesterone content of the corpus luteum rises with the increase in the gland weight. It will be seen that the 26 day pregnant group, represented by solid triangles, shows the greatest range of both gland weight and progesterone content.

## DISCUSSION OF PROGESTERONE LEVELS IN EARLY PREGNANCY.

DISCUSSION OF PROGESTERONE LEVELS IN EARLY PREGNANCY.

The lack of any histological evidence of embryonic degeneration occurring at 16 days in the present study is disappointing in view of the fact that this is the time when great elongation of the blastocyst is occurring. (Winters, Green and Comstock, 1942). It was suggested from the study of Hawk et al. (1955) that the most likely time for embryonic deaths to occur would be between 16 days and the day of return of oestrus.

The blood levels found in both the pregnant and non-pregnant cows at 16 days in this series were of the same order. They also did not differ markedly from the levels at 16 days measured in the six cows during the oestrous cycle. The marked difference in levels at 26 days between pregnant and non-pregnant cows can be explained a return of oestrus as evidenced by the signs of a recent ovulation in the latter group. This is in agreement with a report by Hawk, Wiltbank, Kidder and Casida (1955) who stated that 3 cows out of a group of 50 inseminated animals showed evidence of a quiet ovulation when slaughtered at 34 days.

The slightly higher progesterone levels in both

plasma and corpora lutea in the 26 day pregnant cows over the 16 day pregnant animals is in agreement with the finding of Stormshak et al. (1961) who indicated a rise of progesterone in the corpus luteum during the first few months of pregnancy, followed by a gradual decline. However, Mares and Casida (1960) reported a fall in corpus luteum content at this time.

From the pre-slaughter levels in the pregnant animals between 14 and 16 days the level appears to drop, whereas in the non-pregnant group the levels are either the same or rising slightly. This could indicate a slower maturing corpus luteum in the 16 day non-pregnant group, assuming that blood levels are a reliable guide to luteal levels.

It would appear that at the stages of pregnancy investigated in this study progesterone does not seem to be a critical factor in either conception rates or embryonic death. However, from the findings in the above series together with the levels seen early in the oestrus cycle of the normal cow it would appear that plasma levels of progesterone could be important during the first few days of embryonic life. At this time the fertilised ovum can be

recovered in 90% of inseminated animals (Laing, 1949) but little is known about the position of the fertilised ovum in the oviduct relative to the uterus at this vital stage. It may be that the level of progesterone is critical at the 3 to 5 day stage when, by altering the rate at which the ovum traverses the oviduct, it could affect embryo survival.

In a histological study of corpora lutea during 16 to 34 days of pregnancy in a large series of cows Foley and Greenstein (1958) found a range of corpus luteum weights of 3.20 gm to 9.2 gms which compares with 3.75 gm to 8.60 gms in the present series.

The general relationship of a higher blood level in animals with most progesterone in the corpus luteum or the heaviest gland weight is in keeping with the finding of Moore, Rowson and Short (1960), and Short (1961) who indicated that in superovulated ewes the greater the number of corpora lutea present in the ovaries the higher was the blood level both of progesterone and the 20α epimer, the latter which is also found in sheep plasma.

The two cows in the present series which had twin corpora lutea had blood levels well above the average for

their groups. Cows No. 26 and 40 had levels of 1.41 µg and 1.51 µg where the mean group levels were 1.14 and 1.10 µg/100 ml plasma in the 26 day and 16 day pregnant group, respectively.

In the present study the lack of a close relationship between blood and corpus luteum levels is not surprising as no allowance has been made for the body weight or blood volume of the individual animal. Another factor which could influence this relationship is the vascularity of the corpus luteum and the rate of blood flow through the ovarian vein. Romanoff, Deshpande and Pincus (1962) have demonstrated in the bitch that there tends to be a significant relationship between progesterone secretion rate in ovarian vein blood, and the blood flow rate. Other factors may be involved such as the variation in the rate of metabolism or even the degree of fatness of the animal in view of the fact that fat appears to act as a storage organ.

The effectiveness of progesterone therapy for improving conception rates in repeat-breeder cows is somewhat uncertain. Herrick (1953) and Dawson (1954) both claimed an improvement but the conception rates of

their control animals were rather low. Wiltbank et al. (1956) using two different dosage levels found a 12% improvement over the control animals at 34 days post insemination, but the conception rates were only 42% and 30% in the two groups. In addition where the corpus luteum had regressed there were no live embryos in spite of progesterone therapy. This, together with the fact that an apparently functional corpus luteum was present in all those cows with dead embryos, suggested that the death of the embryo caused regression of the corpus luteum. However, there is the possibility that progesterone therapy could have prevented the death of an embryo and thus maintain the corpus luteum.

Johnston (1958) reported that progesterone treatment improved the conception rates of first service animals but again the controls were very low. The two rates being 72% for the treated and 40% for the untreated. The national figure for conception rates of first service heifers is in the order of 65% (Boyd and Reid, 1961).

Hansel et al. (1960) administered 20 mg of progesterone on the day of oestrus to 36 repeat-breeders with 28 controls. The administration of progesterone at this time is reported to hasten ovulation (Hansel and Trimberger, 1952). However, at 50 days there was no

significant difference in conception rates between the two groups.

Progesterone levels do not appear to differ at 16 days between oestrous cycle cows and cows which have been inseminated, whether pregnant or non-pregnant. This is in agreement with Rowlands et al (1959) who demonstrated that the progesterone concentration in the corpora lutea of mated and unmated guinea pigs was the same at six days after ovulation, which is the mid-point of the luteal phase in the guinea pig.

Staples and Hansel (1961) using an internal marker of progesterone - 4 - C14 estimated the progesterone content of corpora lutea from pregnant and non-pregnant heifers 15 days after mating. They obtained recoveries of 70 to 80% for radio-progesterone and, from the data presented, the average content of progesterone was 210 µg from an average gland weight of 4.2 gm. There was no difference in progesterone content between the pregnant and non-pregnant animals although the 20β hydroxy-progesterone level appeared to be higher in the non-pregnant animals.

In the cow at about 16 days a mechanism must exist which will prolong or terminate the life of the corpus luteum

depending on whether the animal is pregnant or non-pregnant. The developing blastocyst in the cow at 16 days post insemination is a very thin elongated structure which has not yet become attached to the endometrium. It is difficult to imagine how so small a stucture can influence the course of events leading to the maintenance of pregnancy.

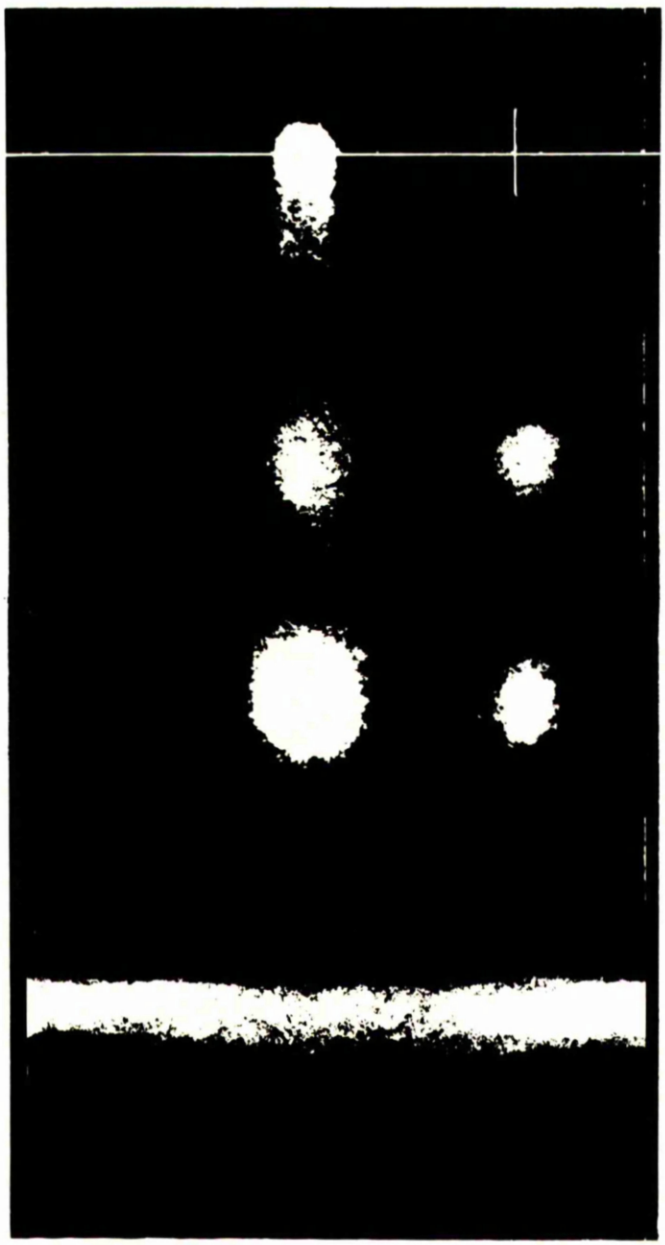
It has been known for some time that the uterus has a profound influence over the corpus luteum. The experiments by Loeb (1923) in the guinea pig demonstrated that hysterectomy at the appropriate time resulted in the maintenance of the corpora lutea in an apparently functional state. Rowlands et al (1959) confirmed these findings and furthermore showed that the corpora lutea so maintained had a higher concentration of progesterone than was seen at any other time.

Similar effects to those seen in the guinea pig were reported for the cow and ewe (Wiltbank and Casida, 1956) and the sow (Du Mesnil du Buisson and Dauzier, 1959) but apparently not in the other species so far investigated (c.f. review Perry and Rowlands, 1961).

Duncan, Bowerman, Anderson, Hearn and Melampy (1961) have demonstrated <u>in vitro</u> the effect of sow endometrium

extracts on progesterone synthesis by homogenates of sow corpora lutea. They found that endometrial extracts taken during the early part of the cycle had some stimulus on progesterone synthesis but that extracts of endometrium from sows at day 16 of the oestrous cycle had a definite inhibitory effect. However, no one has yet shown this effect on the live animal.

FIGURE 3.C.5.




CORPUS LUTEUM COW

MID CYCLE

TABLE 3.C.7.

TOTAL AMOUNT OF 20β HYDROXY PROGESTERONE IN CORPUS LUTEUM IN AT 16 DAYS AFTER INSEMINATION - PREGNANT.
AT 16 DAYS AFTER INSEMINATION - PREGNANT.

| Cow No. | 20β hydroxy progesterone (µg/gland) | Progesterone (µg/gland) | Ratio of 20β to progesterone in gland. |
|---|---|---|---|
| 2 | 37 | 253 | 1:7 |
| 3 | 30 | 337 | 1:11 |
| 4 | 54 | 324 | 1:6 |
| 7 | 49 | 236 | 1:5 |
| 8 | 44 | 309 | 1:7 |
| 9 | 45 | 213 | 1:5 |
| 10 | 66 | 335 | 1:5 |
| 21 | 20 | 300 | 1:15 |
| 29 | 21 | 220 | 1:10 |
| 31 | 36 | 336 | 1:9 |
| 40* | 85 (38 / 47) | 365 (229 / 136) | 1:5 (1:6 / 1:3) |
| MEAN: | 43 | 293 | 1:7.7 |

* Twin corpora lutea.

TABLE 3.C.8.

TOTAL AMOUNT OF 20β HYDROXY PROGESTERONE IN CORPUS LUTEUM

16 DAYS AFTER INSEMINATION - NON PREGNANT.

| Cow No. | 20β hydroxy progesterone (µg/gland) | Progesterone (µg/gland) | Ratio of 20β to progesterone in gland |
|---|---|---|---|
| 1 | 34 | 255 | 1:8 |
| 12 | 42 | 258 | 1:6 |
| 14 | 80 | 332 | 1:4 |
| 23 | 35 | 258 | 1:7 |
| 30 | 41 | 197 | 1:5 |
| 33 | 65 | 298 | 1:5 |
| MEAN: | 50 | 266 | 1:5.8 |

TABLE 3.C.9.

TOTAL AMOUNT OF 20β HYDROXY PROGESTERONE IN CORPUS LUTEUM

26 DAYS AFTER INSEMINATION - PREGNANT.

| Cow No. | 20β hydroxy progesterone (µg/gland) | Progesterone (µg/gland) | Ratio of 20β to progesterone in gland. |
|---|---|---|---|
| 6 | 41 | 331 | 1:8 |
| 11 | 120 | 337 | 1:3 |
| 13 | 38 | 239 | 1:6 |
| 17 | 53 | 454 | 1:9 |
| 18 | 87 | 301 | 1:3 |
| 19 | 150 | 257 | 1:2 |
| 22 | 29 | 323 | 1:11 |
| 26* | 62 (25, 37) | 414 (194, 220) | 1:7 (1:8, 1:6) |
| 35 | 77 | 257 | 1:3 |
| 38 | 75 | 268 | 1:4 |
| MEAN: | 63 | 318 | 1:5.6 |

* Twin corpora lutea.

## 6. 20β HYDROXY PROGESTERONE CONTENT OF THE CORPUS LUTEUM IN THE 16 AND 26 DAY GROUPS.

In most luteal samples it was possible to measure, in addition to progesterone, the amount of 20β hydroxy progesterone. (See Figure 3.C.5.). The calibration curve for progesterone was used to obtain quantitative measurement of 20β "spots" as the ultra violet extinction coefficients of the two compounds in ethanol are essentially the same (Short, 1958[a]). In addition the 20β levels were corrected for extraction losses using the recovery rates obtained with radio progesterone.

A number of 20β levels from the series could not be measured due to streaking from the origin line obscuring and contaminating the 20β "spot" but not interfering with the measurement of the progesterone "spot".

The results for the 16 day pregnant, 16 day non-pregnant and 26 day pregnant animals are shown in Tables 3.C.7., 3.C.8., and 3.C.9. respectively. It will be seen that the mean levels of 20β hydroxy progesterone do not differ markedly among the three groups. However, it will be seen that in all three groups there is a pronounced

individual variation in both the total and relative amounts of 20β hydroxy progesterone and progesterone present. Only trace amounts of 20β hydroxy progesterone were found in the 26 day non-pregnant group.

## DISCUSSION OF 20β LEVELS

Gorski et al. (1958[a]) who first reported the occurrence of the 20β epimer in the corpus luteum of the cow found it to be present in quantities amounting to one third, to one fifth of that of the progesterone present. (Gorski et al. 1958[b]). However, these were from undated samples and no allowance was made for extraction losses.

The quantities of 20β hydroxy progesterone in corpora lutea reported in this study are similar to those reported by Mares et al. (1962), who recorded on the 15th day of the oestrous cycle in the cow 6 µg/gm with a total of 32 µg per gland. The ratio of the latter to progesterone was 1:7.

Bowerman and Melampy (1962) reported 47 ug to 2.0 µg/gm of progesterone and the 20β epimer respectively in day 16 of the cycle. They carried out some incubation experiments in corpora lutea and found that the relative quantities of the two steroids present changed considerably as incubation progressed.

Staples and Hansel (1961) in a study of embryo survival in heifers found much higher levels of the 20β epimer

in the corpora lutea of those animals non-pregnant at 15 days after insemination. Although in the present study there was a slightly higher amount of the 20β epimer per gland in the 16 day non-pregnant group the difference between it and the 16 day pregnant group was not considered to be significant in view of the small number of samples examined.

The exact significance of the 20β epimer in the cow is not clear. Most investigators consider it to be a metabolite as it has been shown to be produced when cow corpora lutea are incubated with progesterone in vitro (Hayano et al. 1954). Apparently, during the extraction procedure utilised in this study none of the progesterone present in the luteal samples was converted to the 20β epimer as radio-activity was not detected in the "20β zone" in several of the chromatograms which were examined.

20β hydroxy progesterone has been shown to have weak progestational activity with certain strains of mice in the Hooker-Forbes bio-assay (Zander et al. 1958), and therefore some investigators add the 20β epimer to the amount of progesterone present and designate this as "total progestin".

Weist (1959) has demonstrated that the conversion

of progesterone to the 20α epimer, by rat ovarian tissue *in vitro*, is in fact reversible but was of the opinion that this interconvertibility was not involved in the mechanism of hormone action. There is no evidence to date that this happens with the 20β epimer.

## SECTION 3 - RESULTS AND DISCUSSION

### D. PROGESTERONE LEVELS IN COWS WITH CERTAIN CLINICAL CONDITIONS.

(1) Cows with hydrops allantois.

(2) Cows with retained foetal membranes.

## D. PROGESTERONE IN COWS WITH CERTAIN CLINICAL CONDITIONS.

### 1. Progesterone in cows with hydrops allantois:

Hydrops allantois in cows generally occurs in late pregnancy and is characterised by an excessive accumulation of allantoic fluid. The foetus and/or the foetal membranes are frequently oedematous.

The first signs are usually gross distension of the abdomen followed by general discomfort and malaise. The incidence of the disease is low but the cow is usually unable to calve and death follows unless the calf is delivered by Caesarean section. Even under these conditions the maternal mortality rate is high possibly due to the sudden loss of allantoic fluid which may amount to as much as 60 gallons. Slow drainage of allantoic fluid from the uterus by means of a retaining catheter, prior to surgical removal of the foetus, appears to be helpful. (Neal, 1956). The cause of this condition is at present being investigated at the University of Glasgow Veterinary Hospital by Mr. P.G. Hignett and Dr. T.A. Douglas with particular reference to biochemical changes in the foetal fluids, the foetus and the dam.

TABLE 3.D.1.

PROGESTERONE LEVELS IN PLASMA AND CORPORA LUTEA FROM COWS WITH HYDROPS ALLANTOIS.

| Case Number | Plasma Progesterone Concentration (µg/100 ml plasma) | Progesterone in Corpus Luteum (µg/gland) | Total Weight of Corpus Luteum (in grams) |
|---|---|---|---|
| 18276 | 1.10 | 96 | 4.9 |
| 18733 | 0.34 | 73 | 2.3 |
| 18734 | 1.09 | 197 | 4.3 |
| 18943 | 0.88 | 181 | 3.7 |
| 19023 | 1.84 | 228 | 7.3 |
| 19606 | 1.21 | 153 | 4.7 |
| 19757 | 0.92 | 120 | 3.5 |
| Means | 0.91 | 150 | 4.38 |

TABLE 3.D.2.

SUMMARY OF HISTORIES OF CASES OF HYDROPS ALLANTOIS

| Case Number | Age of Cow in years | Approximate stage of gestation | Remarks |
|---|---|---|---|
| 18276 | 3 | 7 - 8 months | Calf oedematous and deformed. |
| 18733 | 2 | 8 months | Calf normal. |
| 18734 | 2 | over 7 months | Oedematous membranes - calf normal. |
| 18943 | 3 | over 8 months | Calf normal. |
| 19023 | 5 | 8½ months | Calf normal. |
| 19606 | 4 | 8 months | Oedematous membranes. |
| 19757 | 2½ | 8 months | Calf normal. |

Cases of hydrops allantois occur sporadically and about ten are referred to the Veterinary Hospital per annum. In most cases the affected animals are purchased from the owner so that slaughter and collection of material can be carried out as desired.

The levels of progesterone present in the blood and luteal tissue at the time of slaughter and the history of each animal are shown in Tables 3.D.1. and 3.D.2. respectively. It will be seen that the plasma progesterone concentration shows a wide variation with a range of 0.34 to 1.85 µg/100 ml plasma. The weights and the progesterone content of the corpus luteum are considerably lower than that seen earlier in pregnancy although again there is a considerable variation.

The relationship between plasma levels and gland levels or weights noted in early pregnancy at 16 and 26 days appeared to apply in these hydrops cows. In this small sample there appeared to be a closer relationship between plasma levels and gland weight than the gland content of progesterone.

It will be seen that the average age of these

cases is very low, with a mean age of 3 years while it is apparent that the disease was occurring in late pregnancy at an average time of 8 months, the normal gestation period being 9 months or 280 days.

In the first three cows examined in this series litre samples of amniotic and allantoic fluid were examined for the presence of progesterone and its metabolites but in no case were any of those compounds detected.

## DISCUSSION OF PROGESTERONE LEVELS IN COWS WITH HYDROPS ALLANTOIS.

Plasma levels of progesterone in cows with hydrops allantois were similar to those seen in cows in early pregnancy and at mid-cycle. The only exception appeared to be cow No.18733 which, with a level of 0.34 µg/100 ml of plasma, could have been approaching parturition, as Short (1958[b]) has reported a gradual fall in plasma progesterone concentration 2 to 3 weeks prior to calving. Cow No.19023 had a high plasma level of 1.84 µg/100 ml plasma and from the service date was 256 days pregnant with 24 days until the expected calving date.

The weight of the corpus luteum was generally low with an average weight of 4.4 grams. This agreed with the finding of McNutt (1927) who found low corpus luteum weights in late pregnancy in abattoir cows; although there were two cases in which the glands were heavier than any seen in early pregnancy. It would appear therefore that progesterone levels in the tissues of cows suffering from hydrops allantois are compatible with normality.

## 2. RELATION OF PROGESTERONE TO RETAINED FOETAL MEMBRANES IN THE COW.

In the experiments carried out by McDonald et al. (1952), in which pregnancy was maintained in cows by progesterone injections after removal of the corpus luteum, it was noted that in these cows in which progesterone administration was stopped some weeks prior to the expected parturition date, 7 out of 8 cows retained their placentas, but had a shorter than normal gestation. In a subsequent experiment (McDonald, McNutt and Nichols, 1954) in which the above conditions were adhered to but in which progesterone therapy was recommended for the last 30 days of pregnancy; none of the animals retained their placentas and all calved at or near the expected date. The authors were unable to decide whether the apparently "beneficial" effect of this pre-partum administration of progesterone was due directly to a raised blood level or due to a prevention of early parturition.

In a large scale trial McDonald and Hays (1958) administered progesterone to 93 normal pregnant cows on days 33, 23, 13, 3 and 2 days prior to calving and compared the incidence of retention of foetal membranes in 93 control

RELATIONSHIP OF PROGESTERONE TO RETENTION OF PLACENTA IN THE COW.

| Cow Number | Retention greater than 12 hours | Progesterone Concentration (µg/100 ml plasma) | Remarks |
|---|---|---|---|
| Cochno 1 | yes | 0.21 | Treated Stilboestrol membranes passed at 6 days. |
| Cochno 2 | yes | 0.14 | Treated Stilboestrol membranes passed at 7 days. |
| Quiver 109 | yes | 0.08 | Removed manually on 3rd day. |
| Renton I | no | 0.12 | Membranes passed 6 hours post calving. |
| Renton II | no | 0.23 | Membranes passed 4 hours post calving. |
| Renton III | no | 0.11 | Membranes passed 3 hours post calving. |

Blood samples collected between 12 and 24 hours post calving and prior to any form of treatment.

TABLE 3.D.3.

animals. There was no significant difference between the two groups except that in 3 untreated cows with twins all retained, while in 4 treated cows with twins, none retained their placentas.

In a similar type of experiment, Johnson and Erb (1962) maintained pregnancy in cows, ovariectomised early in pregnancy, with progesterone injections and found that if the injections were stopped more than six days prior to expected parturition then retention of membranes occurred (six out of six cases).

In view of the above findings blood samples were collected from six post parturient cows, three of which had retained their foetal membranes for a period greater than 12 hours post-calving and three of which had delivered them within 12 hours of calving. The progesterone levels are shown in Table 3.D.3. where it will be seen that the levels are similarly low. All blood samples were collected between 12 and 24 hours post-calving and prior to any form of treatment.

## DISCUSSION OF RETAINED FOETAL MEMBRANES

It would therefore appear that there is, in the small sample of cows examined, no difference in progesterone levels in those cows which retained their foetal membranes and those which did not.

The fact that in ovariectomised cows, maintained in pregnancy with replacement progesterone therapy, the occurrence of retention of foetal membranes appeared to be influenced by the presence or absence of progesterone at or near calving, does not necessarily indicate that such is the case in "normal" (non-ovariectomised) cows. The findings of McDonald et al. (1958) in their trial with normal cows would tend to emphasise this. However, from the report of Short (1958[b]) that the progesterone level in the pregnant cow falls slowly from about 2 weeks prior to calving, there is a possibility that the progesterone levels prior to and not after calving could be critical. This is given some support by the findings of Johnson et al. (1962) described above, in which six ovariectomised cows out of six retained their membranes when progesterone administration was stopped more than six days prior to the expected calving date.

The part played by progesterone in the retention of foetal membranes may be associated with uterine motility as Venable and McDonald (1958) have shown that ovariectomised cows as described above have a different post-partum uterine motility from normal cows at this time.

# SECTION 4 - GENERAL DISCUSSION.

## GENERAL DISCUSSION.

Pregnanediol as an index of progesterone metabolism in the cow has been shown to be of no value due to the fact that the excretory pathway of progesterone in this species is probably via the faecal route in the form of an androgen. In this context it is interesting to note that a species of Fusarium which may occur in the rumen can convert progesterone and some related steroids to an androgen (Androsta - 1:4 diene - 3:17 dione).

Bio-assay methods for the estimation of progesterone suffer from a marked lack of specificity, particularly in view of recent findings that a large number of related steroids give positive results in some of the micro-bio-assay procedures. For example, Zarrow, Neher, Lazo-Wasem and Salhanick (1957) have shown that, using a different strain of mice, 17$\alpha$hydroxy-progesterone is 60 times more active than progesterone in the Hooker-Forbes bio-assay. Also the 20 epimers have been shown to have some biological activity in this assay (Zander et al. 1958). Chemical methods have now almost completely superceded bio-assays and it is not surprising that they differ considerably to the results previously obtained by the bio-assay methods.

The plasma and corpus luteum levels of progesterone seen during mid-cycle appeared to be of the same order as the levels seen in cows 16 and 26 days after insemination, which agrees with the finding of Rowlands et al. (1959) for the mated and unmated guinea pig corpus luteum. From the levels reported by a number of workers for the progesterone content of the corpus luteum of the mated and unmated cow a state similar to the findings in the present study exists. This similarity of levels is not surprising as the cow appears to depend almost entirely on the corpus luteum for its endogenous progesterone. Evidence for this is seen in the rapid drop of plasma progesterone concentration described above in the cow, following the removal of either the corpus luteum or the ovaries. This rapid disappearance is common of many species, however, owing to the sensitive techniques used in this study it was possible to measure the low residual levels in these cases.

It is not exactly clear to what degree, if any, the adrenal gland is involved in this finding. However, in view of the finding of a very high level of progesterone in the body fat of cows at this stage in the cycle it is likely that this source may be the more important of the two.

The levels of plasma progesterone in different individual cows examined during this study varied considerably and among the possible factors suggested which might influence these are, the weight and progesterone content of the corpus luteum, the vascularity of the corpus luteum and rate of ovarian blood flow and possibly the amount of progesterone stored in the body fat. In this context it is well known that there is a very wide range of values for the proportion of body fat in different carcases.

The rapid breakdown of the large quantities of injected progesterone in the cows described above would indicate that the liver has a remarkable capacity to catabolise this steroid. It also indicates that there must be a very large turnover or production of this hormone in the cow and it is not surprising that the levels are very low. The plasma levels of "oestrogen" in the cow are also very low. In a recent study Pope, Jones and Wayneforth (1962) were able to detect about 5 µg of "oestrogen" per litre of whole blood but only during the last two weeks of gestation. This is interesting in view of the fact that progesterone concentration is reported to be dropping at this time and might indicate a possible role in parturition.

The above workers were in addition able to detect with some difficulty trace amounts of oestrogen in about 10 litres of blood taken from cows on the day of oestrus.

There is little doubt that progesterone plays an important function in early embryonic life in many species but it was not possible to demonstrate a natural deficiency of progesterone at the times stated in the present study. The promising results obtained by some of the progesterone therapy trials as a means of improving fertility in cattle are difficult to explain. However, the conditions of the trials vary so much between the different groups that no definite conclusion can be reached at present.

It would appear from the studies of progesterone levels during the oestrous cycle in the normal series of cows that the levels early in the cycle might be a more profitable line of approach in embryo mortality studies. However, the levels are so much lower at this time that a more sensitive method of estimation would have to be employed.

Progesterone levels in the clinical cases examined during this study appeared to be compatible with normality.

but in view of the experimental work carried out by McDonald et al. (1952) on retention of foetal membranes in the cow, it would appear that progesterone levels prior to parturition may be worth studying.

# SUMMARY

# S U M M A R Y.

A careful study was carried out of the method of Short (1958[a]) and Rowlands and Short (1959) for the estimation of progesterone in plasma and in corpora lutea respectively. For bovine plasma it was found necessary to acetylate the first chromatogram eluate with an acetic anhydride/pyridine mixture and subject the residue to a further chromatography step. These additional steps increased the specificity of the method for progesterone and at the same time yielded a comparatively pure final product. In addition the incorporation of a radioactive marker to calculate individual recoveries was found to be an advantage in both plasma and luteal samples.

Using the above method plasma progesterone concentration was measured throughout the oestrous cycle in six normal dairy cows. The general pattern consisted of a gradual rise in progesterone concentration from near zero levels on the day of oestrus (day 1) until day 5 when a more rapid rise occurred up to a level of about 1.0 µg/100 ml plasma on day 9. The level remained at about this value until day 16 when a decline occurred until near zero levels

were reached on the subsequent day of oestrus. There appeared to be a considerable variation in the maximum levels attained during mid-cycle in individual cows. The range of values at this time in the six animals used was 0.63 to 1.44 µg/100 ml plasma.

The removal of the corpus luteum or ovaries during mid-cycle in the cow resulted in an initial rapid drop in plasma progesterone concentration followed by a more gradual decline. The fact that this occurred after the removal of either the corpus luteum or the ovaries indicated that the residual progesterone level did not originate from ovarian tissue other than the corpus luteum. It was tentatively suggested that the residual level could be due to a contribution from either the adrenal gland or the body fat depots.

The subsequent determination of comparatively high levels of progesterone in the body fat of cows at mid-cycle emphasised the latter as the more likely of the two sources, with the reservation that an adrenal contribution due to surgical stress could not be ruled out completely.

The rapid disappearance of injected progesterone from the circulating blood of the cow was in keeping with

the observations made in other species. There was evidence to show that this was partly due to catabolism of the progesterone molecule and partly due to uptake by the body tissues, probably the body fat. Furthermore it was shown that renal excretion of progesterone per se did not occur to any appreciable extent. The half life of progesterone in the blood of the cow appeared to be in the order of 6 minutes.

Progesterone in the body fat of cows at mid-cycle was found to be 5 to 10 times that in the plasma and the identification of the progesterone from this source was confirmed by infra-red analysis and gas/liquid chromatography. In fat from cows early in the cycle, from ovariectomised cows and from bullocks, progesterone was not present in any measurable quantity. Progesterone was also found in cows milk where it appeared to reflect the levels found in plasma. However, in spite of milk being more available than plasma, owing to its high fat content, it was not considered to be a better source of material for the study of progesterone metabolism in the cow.

Plasma and luteal levels of progesterone were studied in a series of 40 cows at 16 and 26 days after

insemination. The levels of progesterone in the pregnant and non-pregnant animals when slaughtered at 16 days were of the same order, while at 26 days the marked difference between the levels in pregnant and non-pregnant cows could be accounted for by evidence of a recent ovulation in all cows in the latter group.

In this series of 40 cows there appeared to be a relationship between the plasma levels and both the luteal weight and progesterone content. The higher blood levels appeared to be associated with the heaviest glands which in general contained more progesterone. Conversely, low blood levels were observed with small glands which generally contained less progesterone. The absolute and relative amounts of 20β hydroxy-progesterone did not vary significantly among the groups.

Progesterone levels were investigated in certain clinical cases made available through the Veterinary Hospital, namely cases of hydrops allantois and cases of retained foetal membranes. However, progesterone levels in these animals did not appear to differ significantly from those found in normal cows.

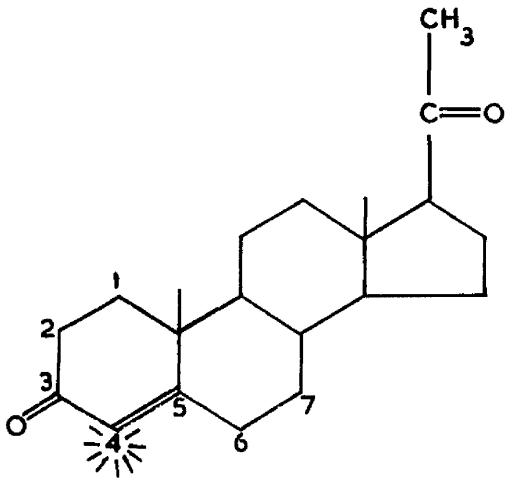
# APPENDIX.

FIGURE AP.1.

# RADIOPROGESTERONE

CARBON ISOTOPE IN POSITION 4

IN STEROID RING




PROGESTERONE—4—C14

SPECIFIC ACTIVITY = 28·3 μCURIES / MGM

$$\text{RECOVERY} = \frac{\text{No. OF COUNTS/MIN. RECOVERED}}{\text{No. OF COUNTS/MIN ADDED}}$$

$$\text{EG.} = \frac{500 \times 100}{1000} = 50\%$$

# APPENDIX

## PROGESTERONE - 4 - C14.

Radioprogesterone contains an isotope of carbon (carbon 14) in position 4 in the steroid ring A. as shown in Figure Ap.1. 0.1 m.c. was purchased from the Radiochemical Centre, Amersham and was contained in 2 ml. benzene in a glass vial. The specific activity of this sample was stated to be 27.9 μ.c./milligram:

∴ amount of progesterone in 0.1 m.c. of this sample

$$= \frac{100}{27.9} \qquad (0.1 \text{ m.c.} = 100 \text{ μ.c.})$$

$$= 3.58 \text{ mg.}$$

∴ number of disintegrations/min from this sample

$$= 3.58 \times 27.9 \times 3.7 \times 10^4 \times 60$$

$$(1 \text{ μ.c.} = 3.7 \times 10^4 \text{ disintegrations/sec.})$$

$$= 221,738,040 \text{ disintegrations/min.}$$

Efficiency of Geiger-Muller tube is in the order of 10% so that only one tenth of the counts will be registered.

Number of counts required = 1000 counts/min. in

0.2 ml. benzene.

∴ final volume required

$$= \frac{221,738,040}{5000 \times 10}$$

$$= 4,450 \text{ ml.}$$

The sample was dissolved in 100 ml of distilled benzene and serially diluted to give approximately 1,000 counts/min in 0.2 ml benzene. This contained approximately 0.2 µ.g. progesterone - 4 - C14.

CHECK ON PURITY OF RADIO-PROGESTERONE

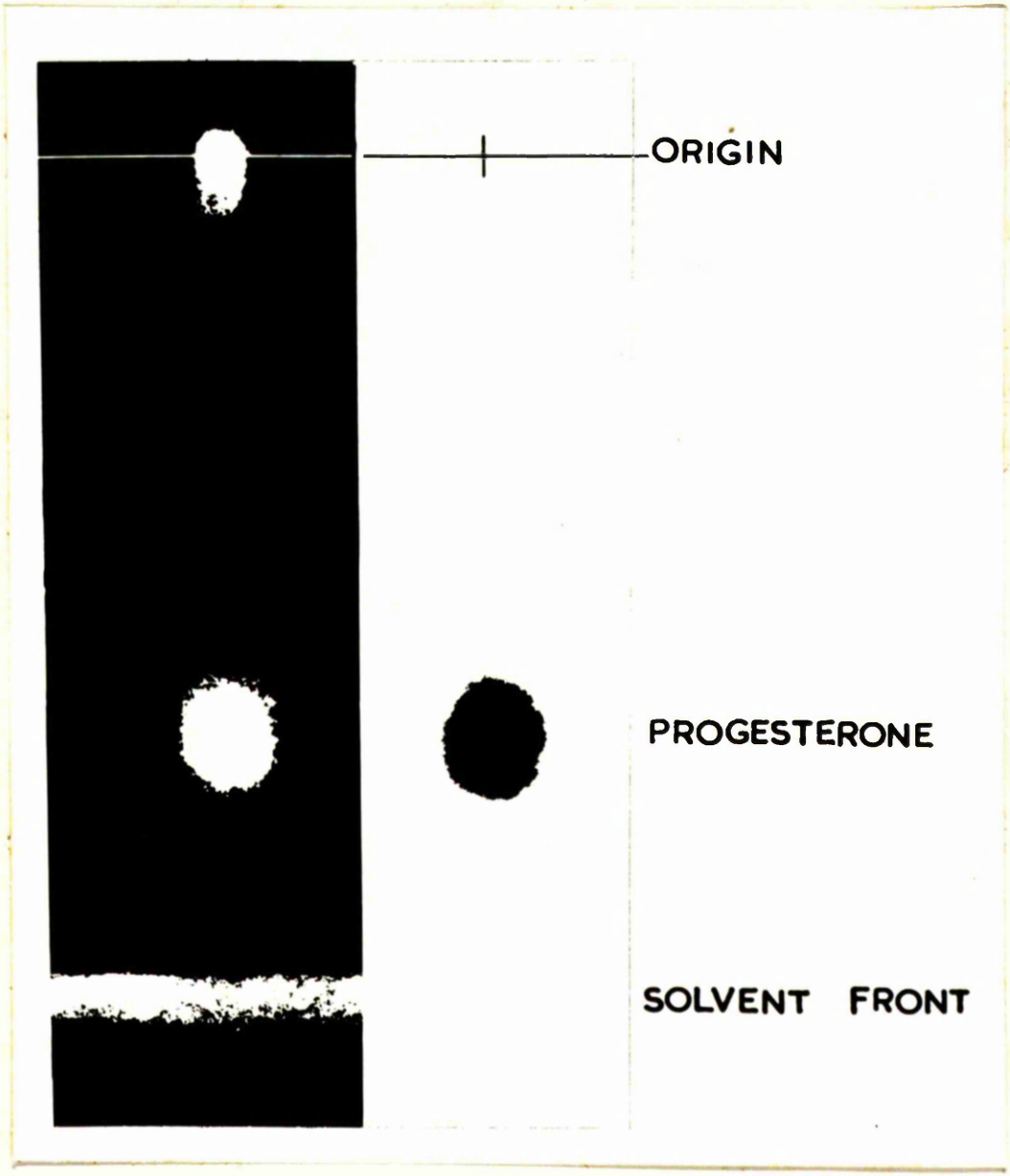
25,000 counts/min. in 5 ml benzene and containing approximately 5 ug radioprogesterone was concentrated and chromatographed in the normal manner. The spot was eluted and indicated a value of 4.60 µg progesterone. The radio activity of the sample was 23,400 counts/min. which represents an approximate recovery of 90%.

This shows that 1000 counts of radioprogesterone is equivalent to $\frac{4.60 \times 1000}{23,400} = 0.19$ µg. progesterone.

This amount was therefore deducted from each corrected reading.

FIGURE AP.2.

AUTORADIOGRAPH OF PROGESTERONE - 4 - C14.



EFFICIENCY OF GEIGER-MULLER TUBE.

As shown above:

Number of disintegrations/min in 3.58 m.g. progesterone - 4 - C14

= 221,738,040

$\therefore$ 1 µg. progesterone - 4 - C14 = $\frac{221,738,040}{3,580}$

= 61,938 disintegrations/min.

From spectrophotometric estimation and counting of progesterone - 4 - C14 it is known that 4.6 µg. radioprogesterone = 23,428 counts/min.

$\therefore$ Efficiency of G.M. tube = $\frac{23,428 \times 100}{4.6 \times 61,938}$

= 8.22%

1 ug radioprogesterone was chromatographed in the normal manner and after contact photography, the paper chromatogram was placed in a cassette together with an X-ray film (unscreened). After 1 week the film was developed and as seen in Figure Ap. 2. the darkening of the film caused by the radioactivity corresponded exactly to the Rf. of the marker progesterone. It will be seen that there is no evidence of radioactivity either at the origin or in the 20 epimer regions of the chromatogram.

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