17

THE ANAEMIA AND HYDRAEMIA OF PREGNANCY IN THE NORWAY RAT, *RATTUS NORVEGICUS*

A thesis submitted for the degree of Doctor of Philosophy of the University of Stirling.

G. T. Maybank,
March, 1971.

ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

C O N T E N T S

CONTENTS (Continued)

FIGURES

I N T R O D U C T I O N

INTRODUCTION

The physiology of pregnancy is a complex and extensive field in which the majority of those working with animals other than man have confined their research and observations to hormones. It is the human subject which has been the most studied in the wider aspects of the general physiological change which occurs during gestation. Following the now classical work on the oestrous cycle of the rat by Long & Evans in 1922, a small number of papers have periodically appeared on various physiological aspects of the oestrous cycle and pregnancy. In 1929 Sure et al noticed that a drop in the concentration of red cells occurred in the blood of the rat on the last day of gestation. They attributed this anaemia to haemorrhage at parturition and to dietary factors. Later work by other authors showed that the anaemia could be attributed to the effects of gestation, particularly the placenta, and was not the product of a deficient diet. Various theories were mooted for the basic mechanism of the anaemia and for its hormonal source, some suggesting an hydration of the blood (hydraemia), thus increasing the plasma volume, others suggesting an increase in blood volume with a disproportionate increase in plasma volume and yet others to a decrease in erythropoiesis. By 1950 the work on this aspect of reproductive physiology had apparently stopped, perhaps because of the greater

interest in hormones. Recently further work has been done on the anaemia of pregnancy in the mouse (Fruhman, 1968; Rugh & Somogyi, 1969).

In recent years analytical techniques have improved and some of the earlier data has become outdated. Furthermore, the improvements in sensitivity and selectivity of the available techniques in the past ten to fifteen years, have encouraged the development of micro and ultra micro techniques. Much of this developmental work has been done to enable studies to be carried out on small organisms such as insects, it did however seem feasible that these techniques could be employed in evolving a method for the serial sampling of small mammals. It was hoped that a technique could be established where-by plasma sodium, potassium and chloride and osmolality could be measured from the supernatant plasma of a single micro haematocrit measurement. In this way it would be possible to observe whether the anaemia was accompanied by a change in these parameters and therefore give an idea of the mechanisms responsible for the anaemia and additionally to study the variation between subjects.

The material presented here falls into two distinct parts: the development of a method for sampling and analysis, and an investigation of the anaemia both experimentally and by a review of the literature.

In this study there are two conventions that have been

used. The first is that the term 'anaemia' implies its simple clinical definition of a drop in the proportion of red cells in the blood, secondly, the nomenclature for the phases of the oestrous cycle will be those of Mandl (1951) abreviated where necessary as follows:-

early oestrus	EO
oestrus	O
late oestrus	LO
early dioestrus	ED
dioestrus	D
late dioestrus	LD

e.g. the phrase "early EO" means the early part of early oestrus. A comparative chart is given of the terms used by various authors (Table 9).

I am extremely grateful for the indispensable help given to me in the analysis of the results by R. R. MacDonald of the Psychology Department. I would also like to extend my grateful thanks to P. Kirk for information and advice, to N. A. A. Macfarlane for his criticism and help and my wife Mary for all the typing she has done. Finally I thank Professor F. G. T. Holliday for his great encouragement, advice and criticism during his supervision of my work. The funds for this postgraduate studentship were supplied by Aberdeen University, The Science Research Council and Stirling University, to which grateful acknowledgement is made.

G. T. Maybank
January, 1971

I declare that the work presented here is the result of my own investigations (unless due acknowledgement has been made) and has not and is not being presented for examination elsewhere.

C H A P T E R I

ASPECTS OF REPRODUCTION

CHAPTER I

ASPECTS OF REPRODUCTION

The Mode of Reproduction

Rattus norvegicus is a spontaneous ovulator but unlike most other spontaneous ovulators, except the mouse and hamster, fully functional corpora lutea are not formed following ovulation. The true luteal phase of the rat's oestrous cycle must be induced by copulation or similar stimuli applied to the cervix, following this, fully functional corpora lutea are formed.

Copulation, whether fertile or not, normally induces a period during which implantation can take place (usually on day 4). During a normal cycle implantation cannot take place nor are diciduomata induceable. If the mating is infertile a condition known as 'pseudopregnancy' occurs. During this period, lasting 12 days, there is an initial phase common to that of pregnancy with the characteristic hormonal uterine changes and weight increases.

The lack of a fully functional luteal phase in the normal cycle is associated with an extremely short oestrous cycle of four to five days. The cycle can be divided into two cytologically distinct phases, Oestrus and Dioestrus. The oestrous (follicular) phase is of approximately 48 hours duration with little variation between animals. The dioestrous phase may vary spontaneously between 2 and 4 days and normally accounts for the different cycle

lengths observed between animals.

Female rats become sexually mature at an age of 10 weeks, the first oestrous cycles are usually lengthened (c. 10 days) but rapidly shorten to the usual length of 4-5 days. The length of gestation is 21-22 days.

The Physiological Effects of the Oestrous Cycle in the Rat

During oestrus the female rat expends appreciably more energy than it obtains in its food, whilst during dioestrus it takes in more food than it needs (Brobeck et al, 1947). Thus it has been found that there were cyclical peaks in weight (up to 10-12 gm) that could be correlated with dioestrus. Corresponding to oestrus, Brobeck et al found that there was a low body temperature, low food intake and high spontaneous locomotor activity. Low body temperatures were consistently noted on the afternoon immediately before the night of hyperactivity (oestrus).

The structure of the uterus in the non-pregnant rat follows a definite cyclical pattern, closely related to the oestrous phase (Long & Evans, 1922; Astwood, 1939; Mandl, 1951). Long & Evans found that towards the end of early oestrus the uterus became distended with fluid, reaching its maximum distension during the early part of oestrus. Thereafter there was regression, with vacuolar degeneration of the epithelium. During early

dioestrus the degeneration was at a maximum then began to regenerate so that by the end of dioestrus the epithelium was fully regenerated. Mating usually occurs during the period of early oestrus, EO, ovulation occurring at about the end of this period (Cooper & Haynes, 1969; Long & Evans, 1922). The unique distension of the uterus by fluid at this time may serve as a medium through which the spermatozoa may swim rapidly towards the oviduct. The fluid was found to support the sperm in a fully mobile condition in vitro for some time (Long & Evans).

The water content of the uterine tissue fell from its maximum in early oestrus to a minimum value late in oestrus at a point in the cycle where ovulation normally occurs (Astwood, 1939), Fig. 1. Distension of the uterine lumen by fluid occurred after the maximum water content of the tissues had been reached, the uterus still remaining distended until the middle of oestrus. It was noted that the fully distended uterus weighed less and contained less tissue water than before distension took place. The peak in tissue water content lasted for 2-3 hours. In pseudo-pregnant rats there was an increase in the percentage of uterine water on day five of the pseudo-pregnancy. Astwood attributed the sudden drop in uterine tissue water to a definite inhibitory mechanism during early oestrus and the period of vaginal cornification. Injection of oestradiol (2.0γ) into intact

FIGURE 1.

Ovarian Venous Progesterone and

Uterine Water Content

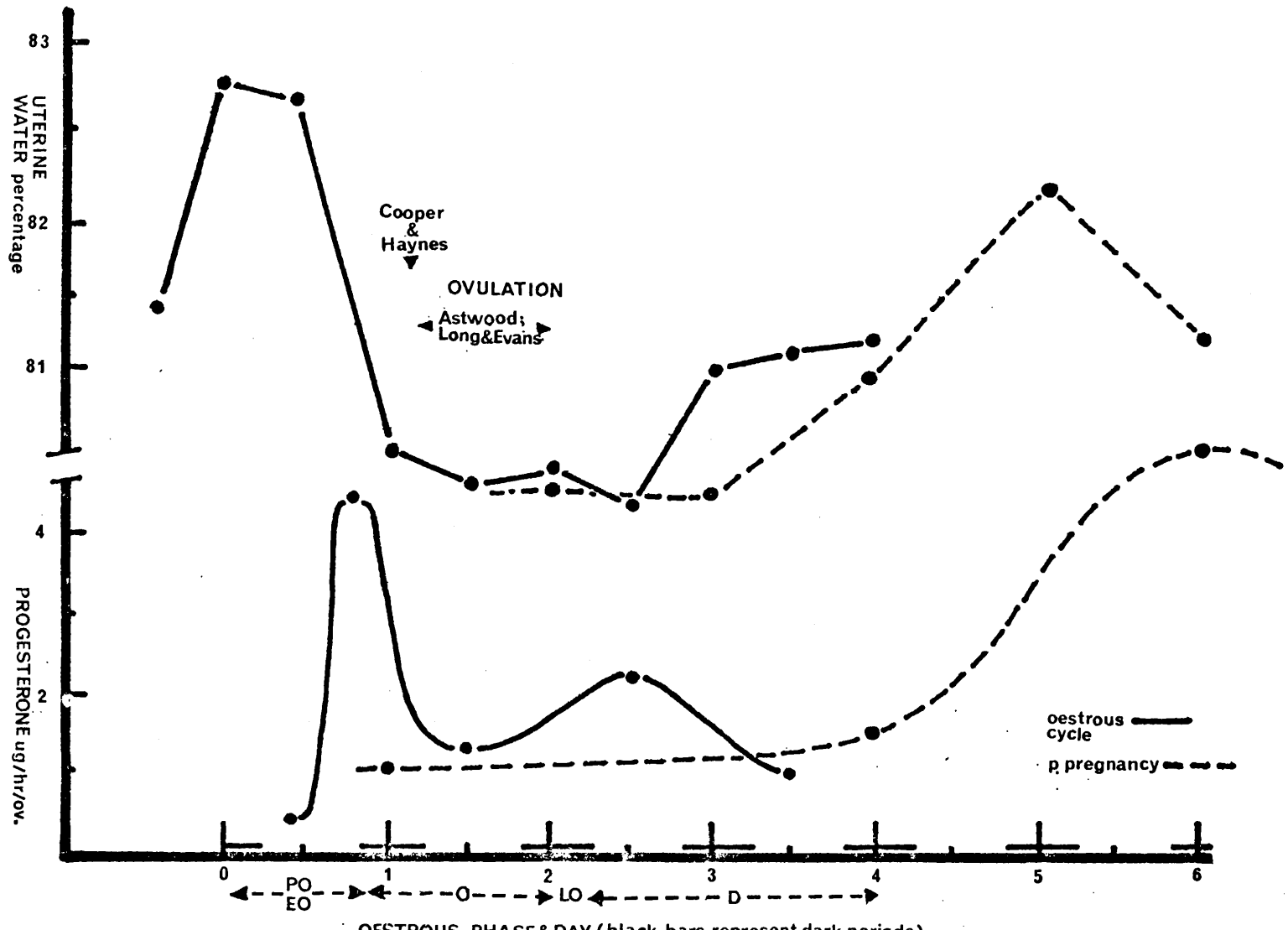


FIGURE 1. OVARIAN VENOUS PROGESTERONE AND UTERINE WATER CONTENT

rats caused an overall rise in the water content but the same pattern of events occurred. Previous experiments (Astwood and Hishaw, cited Astwood, 1939) have shown that this inhibitory effect is a property of progesterone. The data indicated that some substance was secreted by the ovaries during the preovulatory swelling of the follicles which acted upon the uterus in such a way as to reduce the water content and inhibit the action of injected oestrogen. Astwood was very cautious in concluding that this was in fact corpora luteal progesterone, as no evidence had been produced to show that the corpora lutea of the normal cycle were functional. Corpora lutea do in fact secrete some progesterone for a brief time (Zeilmaker, 1963) but soon become atretic. This was supported on histological grounds by Everett (1945). Significantly the secretory rate of ovarian progesterone into the ovarian veins rose rapidly to its highest level in the late afternoon (last stages) of E0, (Hashimoto et al, 1968). A similar peak in progesterone secretory rate occurred on days 5-6 of pseudopregnancy. The blood progesterone level rose sharply after the peak in water content of the uterus had been reached and a rapid fall in water followed, Fig. 1.

During the first 5 days of pseudopregnancy in the rat there was increased oxygen consumption by the uterus (Saldarini & Yochim, 1967). A similarly high oxygen

consumption was found during late dioestrus and early oestrus of the normal oestrus cycle. After ovariectomy, replacement therapy with oestrone and progesterone together gave a similar pattern to that of intact rats. Progesterone given alone merely increased the metabolic activity, but the uterine weight decreased rapidly. Oestrone alone only maintained the uterine weight.

Hamilton et al (1967) found that uterine ribonucleic acid (RNA), protein and wet weight increased during oestrus; the DNA remained relatively constant, thus indicating a cell content increase rather than cell number. From oestrus the RNA content declined to the lowest level of the cycle at dioestrus. At oestrus the increased RNA was found entirely in the cytoplasm.

In an analysis based on more frequent sampling, Heald et al (1970) confirmed the general findings of Hamilton et al but gave a more accurate description of the timing of the events. There were considerable differences between strains; Sprague-Dawley rats showed significant cyclical changes in uterine RNA, water content and DNA, whilst Wistar strain animals showed only changes in RNA content. In the S-D strain the concentration of DNA/unit dry matter was highest in early dioestrus followed by an increase in RNA which reached a maximum at E0/0 (early). The increase in RNA represented an increased synthesis of RNA/cell occurring during this period which was followed by a gradual increase in cellularity, later declining towards

the onset of the next period of EO. A total increase in nitrogen and general growth occurred, reaching a maximum in oestrus (early) some time after the ratio of RNA/DNA reached its maximum.

The blood glucose level rose from 85 to 135 mg/100 ml in the rat in response to a single injection of progesterone (6 ml/100 g), Yang (1970). The dose used in the experiments was large compared to the daily production rate of the hormone during the oestrous cycle in the rat (c. 0.01 mg/hr at early oestrus, (Hashimoto et al, 1968)) but the progesterone level in pregnancy is greatly elevated, the daily dose of progesterone for the maintenance of pregnancy is 1-4 mg.

The Physiological Adaptation to Pregnancy

Weight Change

The maternal weight gain consists of more than a simple increase in the weight of the organs directly connected with pregnancy i.e. the uterus, the foetuses and mammary glands. Experimental evidence suggests that many other discrete organs are affected in some way or another, albeit with possible strain-specific differences.

Abramson (1934) noted significant changes in the following organ weights of Wistar rats during pregnancy:

The eyeballs

Hypophysis

Stomach & Intestines

Liver

Ovaries

Spleen

Suprarenals

Submaxillary glands

Thymus

Thyroid

Uterus

He could detect no significant changes in the weights of the brain, heart, kidneys, lungs or skeleton and musculature but the body length, tail length and head weight showed significant changes. The body weight (corrected for foetuses and uteri) increased significantly in the second half of pregnancy; in the first half there was no significant change.

During pregnancy the female rat gained weight in a characteristic manner. The change in total weight up to half term (days 10 and 11) was gradual but slight, accelerating in the second half to a maximum weight one to two days prior to littering. The weight tended to fall slightly from this maximum value to term (Beaton et al, 1954; Maybank, 1966).

Products of conception The greatest period of weight increase occurred in the last week of pregnancy (Newcomer, 1947; Beaton et al, 1954; Bond, 1948); this period of increased rate of total weight gain was concurrent with the greatest growth rate of the foetuses (Beaton et al, 1954; Csapo & Wiest, 1969). A similar pattern of weight gain by mother and foetuses was found in the mouse (Rugh & Somogyi, 1969).

The uterus showed a gain in weight throughout pregnancy, the greatest increase being during the second half of the gestation period (Abramson, 1934). Placental weight increased rapidly between days 14 and 18 and then remained nearly constant (Csapo & Wiest).

The mammary glands showed an increase in weight from early pregnancy, with the greatest increase from day ten to day 15, thereafter remaining fairly constant in size throughout the remainder of pregnancy and the period of lactation (Folley & Greenbaum, 1947).

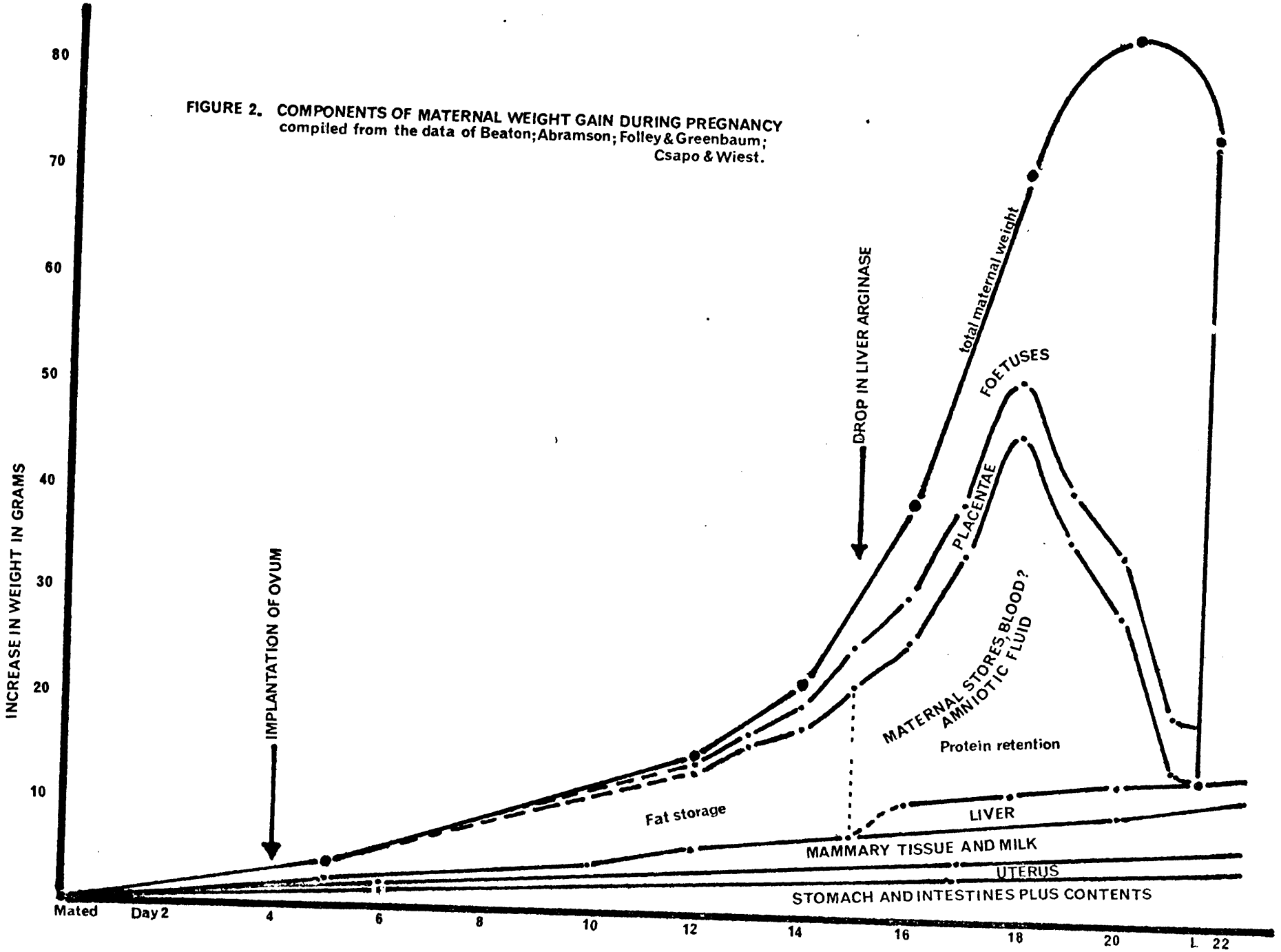
Although Beaton et al did not specify whether the total foetal weight included that of the amnion and fluid, there was a deficit between the total maternal weight gain and that of the foetuses, uterus and mammary glands. Fig 2. Abramson found a significant increase in corrected body weight during pregnancy but not in skeleton weight. The net maternal weight gain could be due to

FIGURE 2.

Components of Maternal Weight Gain

During Pregnancy

FIGURE 2. COMPONENTS OF MATERNAL WEIGHT GAIN DURING PREGNANCY
compiled from the data of Beaton; Abramson; Folley & Greenbaum; Csapo & Wiest.



(i) an increase in fat or carcass protein, (ii) an increase in discrete organ weights, or (iii) an increase in body fluids.

Organ weights Abramson found that many organs not directly concerned with pregnancy showed a change in weight. Corresponding to the general increase in body weight, there was a great increase in liver weight. The ovaries showed an increased weight in the second half of gestation and the spleen a sudden increase in the first half followed by a decrease to normal by term. Until almost mid-pregnancy the corpora lutea were not different in character to those of ovulation, but after day 10 continued slow growth permitted them to attain dimensions never found in corpora lutea of ovulation, mostly by growth of the lutein cells (Long & Evans, 1922). In contrast to the condition found in humans (Hytten & Leitch, 1964) the thyroid showed a significantly lowered weight throughout pregnancy. It has been noted that the orbital content of the eye increases notably by water uptake and fat accumulation under the influence of thyroid stimulating hormone, comparable water and fat accumulations occur elsewhere in the body (Prosser & Brown, 1961).

The change in liver weight noted by Abramson (1934) was also found by Folley & Greenbaum (1947) and Beaton et al . Folley & Greenbaum considered it probable that the increase in liver weight in late pregnancy was above that

which was due to the general body growth of the mother.

The weight increase due to organ weight changes is shown (Fig 2.). From this it can be seen that a substantial part of the maternal body weight increase from day 8 to day 20 is still unaccounted for. This can be due to increases in fat, protein or body fluids.

Body Composition Changes During Pregnancy

Beaton et al, (1954) found that during the first two weeks of pregnancy in the rat there was a marked storage of fat and water in the maternal carcass, whilst there was little foetal growth. From day 15 there was a definite and sharp decrease infat stores with increased retention of protein, Fig. 2. The time of decrease in fat storage and increased nitrogen retention occurred at the commencement of rapid growth of the foetuses and placentae (Beaton et al; Csapo & Wiest). It was suggested that there might have been an inter-relationship between fat and protein metabolism, the fat being catabolised to provide energy and thus conserve the protein at a time when protein storage was desirable. Water was retained throughout gestation but was rapidly excreted following parturition. Pike et al (1954) noted that unless the diet was deficient it was during the third week of gestation that retention of nitrogen reached its highest levels.

The pattern of fat deposition found by Beaton et al

from day 0 to 14 corresponded very closely to the progesterone levels found in the ovarian, venous and peripheral blood, the peak of progesterone concentration occurring at 14 days with a marked and steady decline from this point to day 19, (Hashimoto et al, 1968; Csapo & Wiest, 1969; Raj & Moudgal, 1970), Fig.6 . Galetti & Klopper (1962) found that female non pregnant rats treated with 3.14 mg progesterone daily showed an increase in fat content from 12.3% to 15.8% of the total body weight. The distribution of the fat deposits was not affected.

Progesterone and TSH are not the sole hormones having an effect on fat stores. Growth hormone (GH) has been shown to promote general protein deposition at the direct expense of fat stores in fully grown female rats (Greenbaum, 1953). GH caused a rapid mobilisation of lipid to the liver, the changes occurring mainly in the neutral fat fraction. The hormone also stimulated the direct extra-hepatic utilization of fat (Greenbaum & McLean, 1953 a & b), the total fat oxidation being greater than the maximum oxidation rate of the liver - the other sites were considered to be heart muscle, kidney, spleen and lung. Although oestrogen enhanced GH release in humans (Frantz & Rabkin, 1965) there is no data at the moment to suggest that this is the case in the rat. Human GH (HGH) did not change significantly between the proliferative and secretary phases of the menstrual cycle but rose at

ovulation and premenstrually (Spellacy et al, 1969); increased GH levels have not so far been found during pregnancy in the rat (Schalch & Reichlin, 1966). Vasopressin can stimulate GH release in the human (Gagliardino et al, 1967)

The Anaemia & Hydraemia of the Rat in Pregnancy

The anaemia during pregnancy indicated by the fall in haematocrit throughout the later part of gestation noted initially by Sure et al (1929) and by Beatom et al (1954) has been studied more extensively by Van Donk et al (1934), Newcomer (1947) and Bond (1948). Rugh and Somogyi (1969) and Fruhman (1968) have conducted similar experiments on the mouse.

Van Donk et al, in a comprehensive cross sectional study, found a fall in red cell volume during pregnancy. This anaemia became most pronounced after the 16th day and returned to normal by the 12th day post partum. The overall changes were a fall from a haematocrit value of 50% to 45% in the first 16 days and a further fall to c. 37% at parturition. Red cell count and haemoglobin followed a similar pattern whilst whole blood water showed an increase which followed the haematocrit inversely, Fig. 3. There was no evidence to suggest that the changes in the blood were influenced by the variations in diet but rather that they were due solely to pregnancy. The authors have

FIGURE 3.

Prepartum Changes in Blood Composition

FIGURE 3. PREPARTUM CHANGES IN BLOOD COMPOSITION

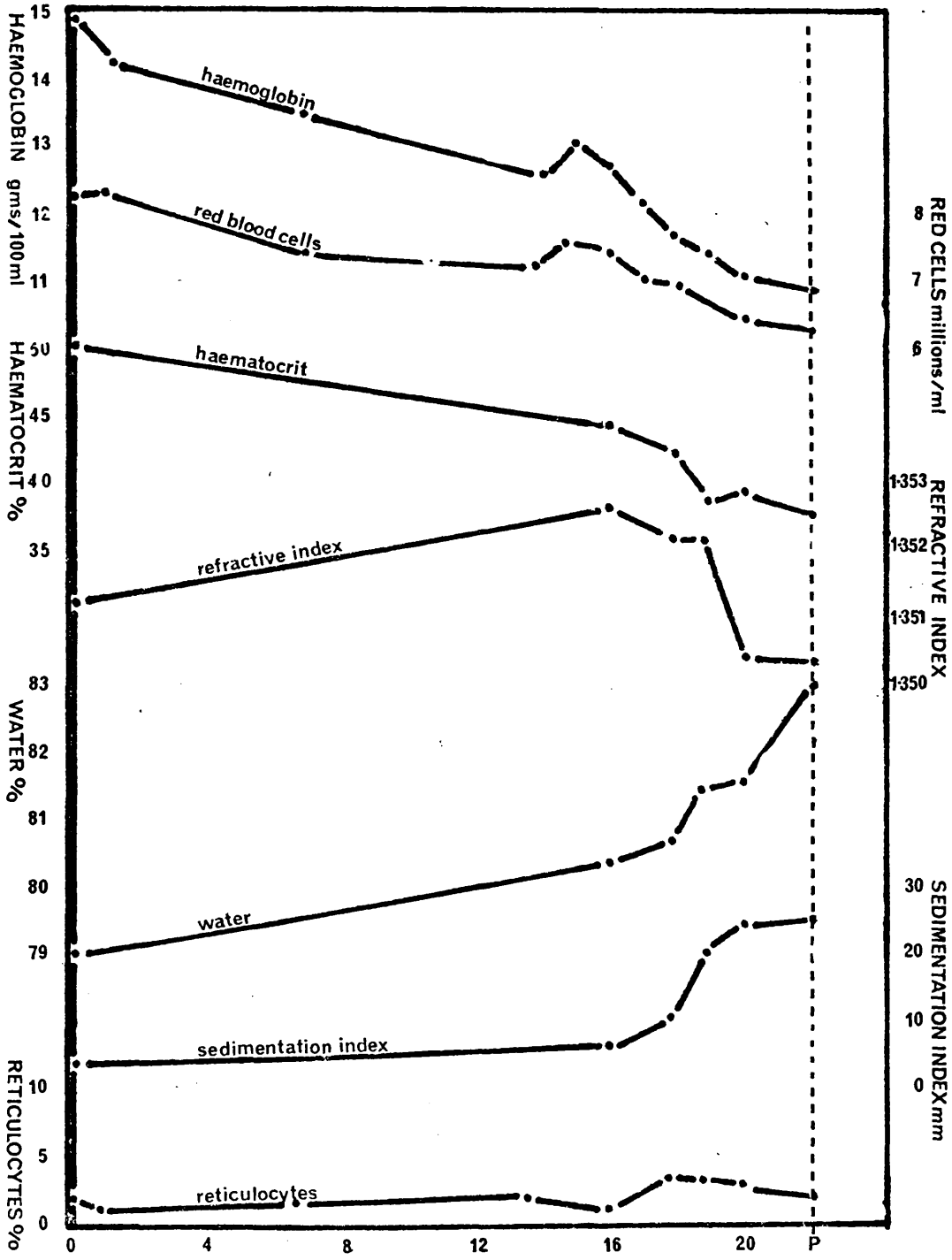


figure from VanDonk et al (1934).

unfortunately not used plasma or serum but whole blood for the water analysis and by simple proportion, the water content of whole blood will increase by 2-3% solely due to the drop in the red cell percentage. Thus as evidence for an hydraemia the figures quoted (3.5% increase in blood water) must be treated with caution. Better evidence for a dilution of the plasma by water was given by their figures for refractive index, Fig. 3. These indicated an initial concentration of the plasma solids and then a dilution; in addition, the sedimentation rate showed a continuous and large increase (580%).

During a normal pregnancy there was a decided diminution of the red cell count and haemoglobin per unit volume of peripheral (tail) blood (Newcomer, 1947). The effect became most noticeable on the 13th day and decreased progressively until the last day of pregnancy - the haemoglobin and red cell count fell in parallel. The anaemia normally disappeared approximately one week after parturition. The constancy of the colour index* and the fact that the anaemia disappeared relatively rapidly, combined with the findings of Sure et al and Van Donk et al led Newcomer to the conclusion that it might be a simple dilution of the blood with water - an hydraemia.

$$\text{*Colour Index} = \frac{\text{concentration haemoglobin exp. blood}}{\text{concentration haemoglobin normal blood}} \cdot \frac{\text{Red cell count exp.}}{\text{Red cell count normal}}$$

Bond (1948) found an almost identical pattern of events during pregnancy to that found by Newcomer, but in addition he studied the blood volume and was thus able to calculate that the absolute numbers of erythrocytes and amount of haemoglobin increased throughout pregnancy but not to the extent that the plasma volume increased. The absolute numbers and haemoglobin increased more rapidly in the last seven days, Table 1. The anaemia that occurred during pregnancy in the rat was considered by Bond to be apparent rather than real, a phenomenon which he termed a 'physiological anaemia'.

The increase in blood volume parallelled the total (maternal and foetal) body weight throughout the period of gestation. There was a rapid loss in blood volume post partum, without an appreciable change in erythrocyte and haemoglobin levels from those of day 22 (Bond, 1948). The specific gravity of the blood also fell in the last week.

The determination of the blood volume (Bond, 1948) was made with a vital red dye (Trypan red). He did not state whether he diluted the standards with plasma or water or whether he protected the blood sample effectively against haemolysis. These factors are of great importance especially when using a red dye, the possible error being of the order of 20-30%, (Gregersen & Rawson, 1959). The protein content of the plasma falls throughout

Table (1) - Observations on the Blood of Female Rats at Three Stages of Pregnancy

	1st day	13th-14th day	21st-22nd day[1]
Body weight (gm)	200 ± 8[2]	230 ± 8	261 ± 9
Erythrocytes (million/cmm)	8.84 ± 0.21	8.04 ± 0.15	7.62 ± 0.19
Haemoglobin (gm/100 cc)	14.2 ± 0.2	13.8 ± 0.4	13.2 ± 0.2
Colour Index	0.99 ± 0.02	1.05 ± 0.02	1.07 ± 0.02
Haematocrit (%)	42.9 ± 1.3	40.0 ± 1.2	38.6 ± 0.6
Specific gravity whole blood	1.0573 ± 0.0004	1.0566 ± 0.0007	1.0541 ± 0.0003
Specific gravity plasma	1.0280 ± 0.0004	1.0284 ± 0.0004	1.0266 ± 0.0004
Blood volume[3](cc.)	17.34 ± 0.55	20.99 ± 1.09 (5)	24.45 ± 0.72
BV/100 gm body weight	8.2	8.0	8.2

1 Values for P for the determinations at the 21st-22nd day stage are less than 0.01 except for colour index and plasma specific gravity where $P < 0.02$

2 Mean value and standard error; each figure represents determinations on 9 animals except the number appears in parentheses.

3 Blood volume from rats of Group 3 in which only blood volumes were determined. The average body weights on days 1,14, and 22 of pregnancy were 212 ± 6,260 ± 6 and 299 ± 8 gm respectively.

Table from Bond (1948)

pregnancy (Beaton *et al*; Sure *et al*) and this could have enhanced the apparent haemodilution. Bond's figures for blood volume must therefore also be treated with caution.

Rugh & Somogyi (1969) in work on the pregnant mouse found a similar pattern for the anaemia to that in the rat. The anaemia became apparent earlier (day 5-6) i.e. immediately following implantation at about day $4\frac{1}{2}$, and occurred despite accelerated erythropoiesis. During anaemia the white blood cell counts of the mouse showed a dramatic change. The leucocytes increased to well above control levels on days 2-3 and 9-10, at other times remaining well below the control level. Sure *et al* (1929) found no change in total leucocyte levels during gestation, at delivery, or during lactation in the rat. In the mouse the early rise at day 3 might have been due to (i) the movement of the morula-blastula into the uterus causing local leucocytosis and (ii) a slow reaction by leucocytes to sperm cells in the reproductive tract (Rugh & Somogyi). The peak of white cells at 10 days coincided with very active placenta formation. Histological sections of the uterus at day 3 showed a massive migration of leucocytes towards the lumen and phagocytosis of the spermatozoa.

During early pregnancy the maternal spleen showed a definite increase in size in the rat (15%), followed by a loss in weight in the second half to a weight only

slightly greater than in non-pregnant controls (Abramson). The mouse spleen showed striking changes during early pregnancy with a marked increase in erythropoietic activity dropping to, or below, normal levels in the last four to five days of pregnancy (Fruhman, 1968). In the mouse the maternal spleen was considered the primary organ of erythropoiesis (Fruhman). This was in contrast to the dog spleen which exhibited a definite shrinkage during pregnancy (Bancroft & Stevens, cited Fruhman). In the dog, the spleen, besides being the site of lymphocyte and monocyte formation, acts as a contractile reservoir for erythrocytes. These are released into the blood stream in response to a sudden increase in activity or during pregnancy when there is an increased demand for oxygen transport (Samson Wright, 1966). In the majority of adult animals however, the spleen, although acting as a limited reservoir for erythrocytes, is primarily an organ concerned with the formation of lymphocytes, plasma cells, antibodies and the destruction of red blood cells (Ham, 1965).

The results of Rugh & Somogyi suggest that implantation and placentation may have an effect upon the appearance of the anaemia in the pregnant mouse. In the rat Newcomer found that placental tissue in the absence of pituitary, foetuses and ovaries produced an anaemia almost as severe as that of pregnancy. Neither the ovary nor the living foetuses seemed to have any direct effect upon

the production of the anaemia. It was considered possible that the foetuses, being necessary for the full development of the placentae, may have had only an indirect effect upon the anaemia. The pituitary seemed to exert an inhibitary effect upon the anaemia but was overidden by the placenta (the pituitary tending to increase the red cell numbers). It was considered possible that placental oestrogen might have been responsible for the anaemia, oestrogens having been shown to exhibit an anaemia-producing effect. Oestrogen levels rise in the plasma towards term and output from the ovaries begins again at day 14 (Raj & Moudjal, 1970; Yoshinaga et al, 1969).

Blood Volume and the Plasma Composition During Pregnancy

Plasma proteins fell during pregnancy (Beaton et al, 1954; Sure et al, 1929) despite increased nitrogen retention in the third week (Pike et al, 1954). Blood urea, although raised during lactation, showed no obvious increases above the normal fluctuations during gestation (Parsons, 1930). Beaton et al found a fall in blood amino-nitrogen during the last week of gestation but blood urea showed no change except at parturition when there was an elevated level. It was suggested that the decreased blood amino-nitrogen might be a reflection of the increased utilisation of amino acids by the foetuses.

If plasma proteins fall there will be an accumulation of fluid isosmotic to the plasma in the interstitial space

(Yamamoto & Brobeck, 1965). This will lead to an increase in the total extracellular space (sodium space) above that of the blood volume increase. Such an increase of extravascular fluid would constitute a pitting oedema. A reduction in plasma proteins should also increase the glomerular filtration rate (GFR). There is little in the literature available on oedema other than in the human.

The absolute blood volume increased by 40% during pregnancy but in parallel with total body weight (maternal and foetal) BV/100 gm = 8.2 - 8.0 (Bond), Table 1. Bond used an inaccurate single dilution technique (Trypan red) which to some extent invalidates his results. Bond's values for the non-pregnant controls (7.8 - 7.6 ml/100 gm body weight) are higher than recent and accurate combined plasma dilution and cell tagging methods have given, Table 2.

Table (2) - Blood Volume of the Normal Rat

Volume (ml/100 gm body weight)	Method	Author
7.6 - 7.8	Trypan Red dilution technique	Bond (1948)
5.6 - 7.0	T-1824 plasma dilution and P^{32} cell tagging	Biol. Data (1964)
5.7	dye and cell tagging	Prosser & Brown (1962)
6.0	not stated	D'Amour & Blood (1965)

Lichton (1961) found an increase in extracellular space (measured as SCN, thiocyanate space), at term in pregnant rats and a slight decrease in serum sodium and serum osmolality. Fruhman (1968) found an increase in the plasma volume of the pregnant mouse at term (1.65 ± 0.045 ml non pregnant, 2.96 ± 0.081 ml pregnant, $P < 0.01$).

During the course of pregnancy there was a net accumulation of sodium by dam and foetuses during the second and third weeks. This closely paralleled the weight gain (Lichton). The greatest accumulation occurred in the last third of gestation, i.e. the period of rapid foetal growth (Pike et al, 1954). The uterus and contents showed a mean sodium content of 5.22 ± 0.44 mEq at term which was not significantly different to the 6.56 ± 1.26 mEq gained by the dams and foetuses.

At term the total SCN space was 37.3 ± 0.96% (v/w) of the body weight compared with 31.2 ± 0.59% of body weight in the non-pregnant controls (Lichton). The amount of sodium added to the extracellular fluid (dams and foetuses and amniotic fluid) of 16 animals in the last third of gestation was 3.32 ± 0.65 mEq compared to 3.27 ± 0.67 mEq gained overall by dams and foetuses during the same period i.e. all the sodium accumulated in this period appears to have been accommodated in the expanded extracellular space of dams and foetuses. This does not however necessarily imply an increased blood volume for the dam.

Of particular interest was the fact that the absolute amount of sodium saved by the dams was not significantly different from the total amount of sodium present in the foetal tissues. The foregoing consideration plus the fact that there was no significant post-partum loss of sodium suggested that there was no net accumulation of sodium in the maternal tissues during pregnancy (Lichton, 1961). At term there is little indication of a greatly expanded maternal vascular system, Fig. 2., although the possibility of an expanded maternal blood volume (Bond,1948) with no net accumulation of sodium by the dam (Lichton, 1961) could result in a lowering of the extracellular fluid (including the blood plasma) sodium of the dam. Two facts from Lichton (1961) indicate that this could be the case:-

1. serum osmolality is lower at term
2. serum sodium is lower at term

- see Table 3.

Table (3) - Serum sodium, Osmolality and SCN Space at Term

	No of Rats	Serum Na^+ mEq/l	Serum Osmolality	SCN space % body weight
Non pregnant*	6	140.5 ± 0.68	297.2 ± 0.84	31.2 ± 0.59
Pregnant	12	134.5 ± 1.10	285.8 ± 1.67‡	37.3 ± 0.96‡

* Mean ± SEM ‡ Significantly different from non-pregnant at 1% level of confidence

Table from Lichton (1961)

Post partum the non-lactating dams tended to retain further amounts of sodium indicating a possible depletion of sodium stores in the maternal tissues prior to littering. Simultaneously with the depressions in serum osmolality and serum sodium content Lichton also noticed a relative antiduiresis, without an accompanying anti-natriuresis near term, having administered an oral isotonic saline load. The question of antidiuresis and oliguria during pregnancy was studied in a later paper, (Lichton, 1963). It was found that on day 13 there was enhanced excretion of water, sodium and total solutes, and on day 20 a depressed excretion proportional to the number of new born pups. The results were obtained from isotonic saline loading (5% v/w body weight) on the same group of rats at 6 days, 13 days and 20 days, Table 4. In order to determine whether these changes were due to altered glomerular filtration rate (GFR), the GFR was measured by inulin clearance in a further group of animals under the same conditions of saline loading prior to mating and at 13 days and 20 days of gestation, Fig. 4. and Table 5. Whether taken on an absolute or body weight basis the variations in GFR showed little relation to the pattern of urine excretion, Table 5. Thus on an absolute basis the GFR rose significantly on day 20 while urine flow, sodium excretion and total solute excretion fell, Fig. 4. Lowered blood protein towards term (Beaton et al, 1954)

FIGURE 4.

GFR, Urine Flow and Blood Protein

During Pregnancy

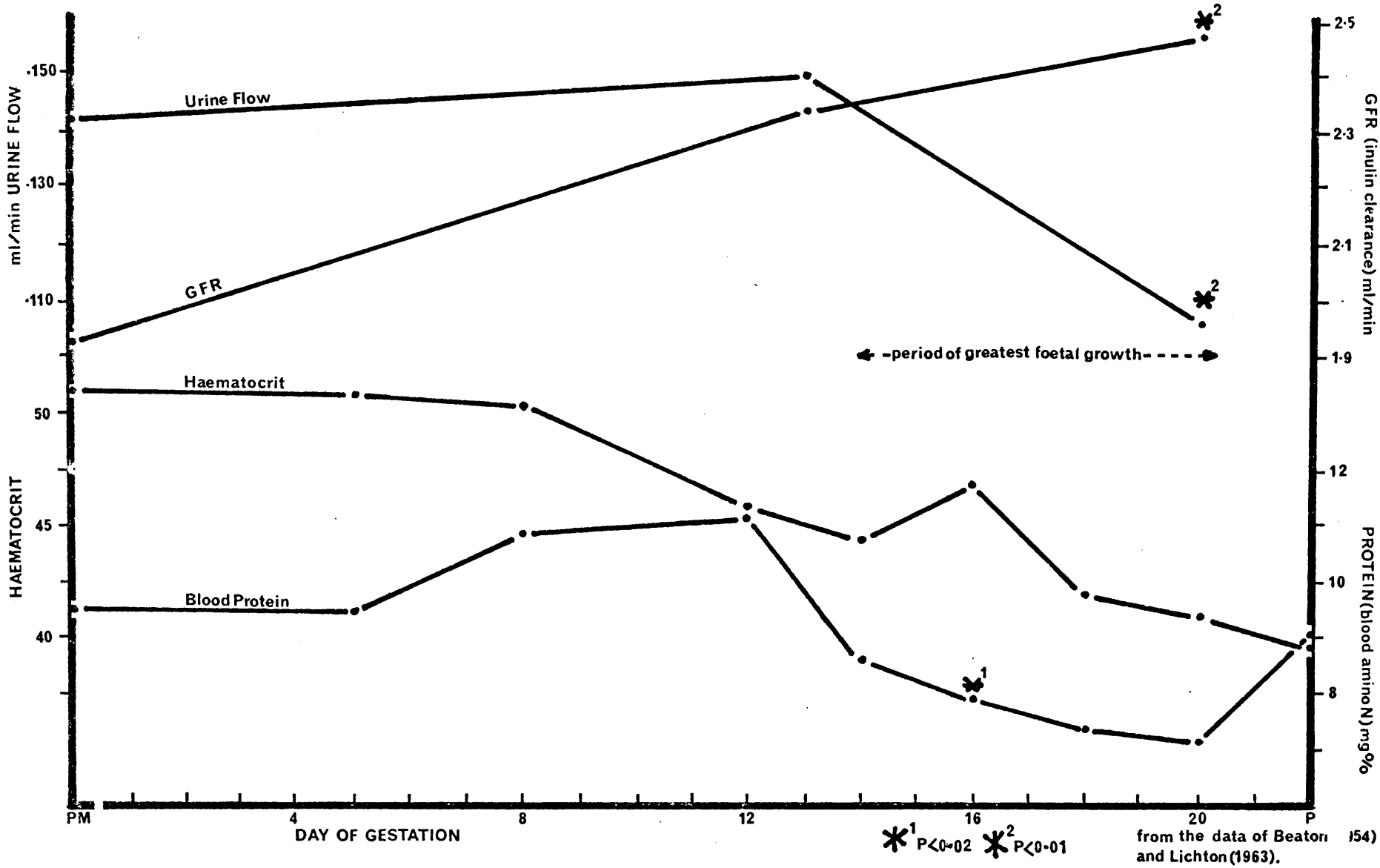


FIGURE 4. GFR, URINE FLOW AND BLOOD PROTEIN DURING PREGNANCY

Table (4) - Excretion of water, sodium and total solutes in 6 hr after administration of isotonic saline solution to conscious rats

	Body Weight	Water		Sodium		Total Solutes	
		ml	ml/100 g	mEq	mEq/100 g	mOsm	mOsm/100 g
Nonpregnant	224±3.0	9.8±0.32	4.36±0.12	1.46±0.05	0.65±0.019	4.86±0.17	2.16±0.07
Pregnant 6 days	232±3.7	10.7±0.51*	4.63±0.20	1.42±0.10	0.64±0.033	5.07±0.34	2.28±0.11
Pregnant 13 days	249±318	12.7±0.51*	5.08±0.17*	1.79±0.08*	0.73±0.030	6.48±0.25*	2.58±0.09*
Pregnant 20 days	260±4.4	9.8±0.68	3.65±0.26	1.34±0.07	0.50±0.029*	4.50±0.25	1.69±0.10*

Values are means ± SEM, 23 rats

*Significantly different from nonpregnant at 1% level of confidence (Student's method for paired differences).

Table from Lichton (1961)

Table (5) - Summary of renal clearance measurements in rats anesthetized with sodium pentobarbital

Unit of measure	Nonpregnant	13 days pregnant	20 days pregnant
No of rats	12	10	10
Body wt., g	224±2.1	252±2.9	303±8.1
	Inulin clearance		
Ml/min	1.92±0.07	2.35±0.22	2.48±0.18*
Ml/min/100 g	0.856±0.03	0.926±0.08	0.824±0.06
	Urine Flow		
Ml/min	0.142±0.005	0.150±0.008	0.106±0.014
Ml/min/100 g	0.063±0.002	0.060±0.003	0.036±0.005
	Excretion of water, %		
	7.40±0.36	6.73±0.52	4.32±0.53*
	Na^+ excreted		
µEq/min	16.9±0.74	17.7±1.24	14.4±1.82
µEq/min/100 g	7.50±0.39	7.02±0.46	4.84±0.66*
	Excretion of filtered Na^+, %		
	6.63±0.41	5.79±0.36	4.40±0.50*
	Total Solutes excreted		
µOsm/min	44.5±1.31	51.0±3.10	42.2±3.93
µOsm/min/100 g	20.0±2.11	20.2±1.12	14.1±1.48
	Excretion of filtered solutes, %		
	8.06±0.44	8.03±0.49	6.20±0.48*
	T^cH_2O		
Ml/min	0.014±0.004	0.032±0.006	0.047±0.008*
Ml/min/100 g	0.0061±0.002	0.0124±0.002	0.0155±0.002*

Values are means ± SEM

* Significantly different from nonpregnant at 1% level of confidence (t test for comparison of means).

(T^cH_2O, free water reabsorption)

Table from Lichton (1961)

would lead to an increase in GFR (GFR = hydrostatic pressure of blood - colloid osmotic pressure of plasma protein (Prosser & Brown, 1962)). On day 13 however there was enhanced GFR with enhanced urine flow, sodium excretion and total solute excretion (Lichton, 1963). The results for day 20 indicated an increased tubular reabsorbtion of both water and solute. Although a number of hormones might have had a similar combined effect (vasopressin, oestriol and progesterone), the hypothesis was put forward that the decreased flow of urine and excretion of solutes in response to acute saline loading late in pregnancy (day 20) was a result of a relative depletion of the extracellular fluids of the dam as fluid entered the foetal tissue. Possibly as a result of excitation of the volume receptors this depletion led to conditions favouring increased tubular reabsorption of filtered water, sodium and total solutes.

The enhanced absolute GFR on day 13 with increased urine flow and associated solutes was ascribed to an expanded maternal extracellular space at a time when the foetuses were not accumulating large amounts of fluid at the expense of the dam. The exaggerated decreases in excretion by the rats with the most foetuses could have been a consequence of a tendancy for greater amounts of maternal fluids to be transferred into the foetuses.

Lichton's (1961) work on SCN space involved the removal of a large preliminary blank sample of blood (1 ml).

This is c. 8% of the total blood volume for a 200 gm rat and it could have had an effect on the osmolality and electrolyte content of the plasma. During the experimental period, after the removal of the blank sample, the rats received no water and the reaction to the volume change could have differed at different stages of gestation. In humans thiocyanate does not equilibrate evenly throughout the maternal body fluids and the product of conception (Hytten & Cheyne, 1962).

Paaby (1959) has shown that there was a slight change in plasma and serum water during gestation in humans. His studies were longitudinal (serial sampling, same subjects) and indicated a certain amount of individual variability, but in all subjects a sharp drop in plasma water took place before parturition. This is the opposite to the changes found in the rat where the general impression is of a dilution of the plasma or serum at term. During gestation Paaby noted cyclical changes which corresponded closely in length to the non-pregnant menstrual cycle length for each subject. These cycles have never been demonstrated in the non-pregnant woman (cited Paaby, 1959). Paaby (1961) in a statistical evaluation of the changes occuring in the plasma/serum water content concluded that the slight rise and fall found during pregnancy must be treated with caution as their statistical significance was very slight.

Evidence has been given to show the existence of an anaemia during pregnancy in the rat, that there may be a haemodilution of the red cells by plasma and possibly a dilution of the plasma by water, or an hydraemia.

Hormonal States During the Oestrous Cycle & Gestation

It is not the intention to develop theories for the hormonal mechanisms of ovulation and gestation. These have recently been ably discussed by Gorski (1968), Rothchild (1965) and Raj & Moudgal (1970). What is necessary for the present study is an accurate picture of the hormonal states of the blood plasma at different phases of the cycle and the effects these hormonal titres may have on the plasma water and electrolytes.

The Oestrous Cycle

Gorski's (1968) theory of ovulation implicates oestrogen and luteinising hormone (LH) as the two hormonal principles responsible for ovulation. Although progesterone is high near ovulation this is a secondary effect, the level of plasma progesterone being dependent on the combined activity of LH and LTH (luteotropic hormone, prolactin in the rat). Oestrogen is considered to be bi-phasic in its stimulation of luteinising hormone releasing factor (LRF); at low plasma titres it stimulates LRF (and therefore increases plasma

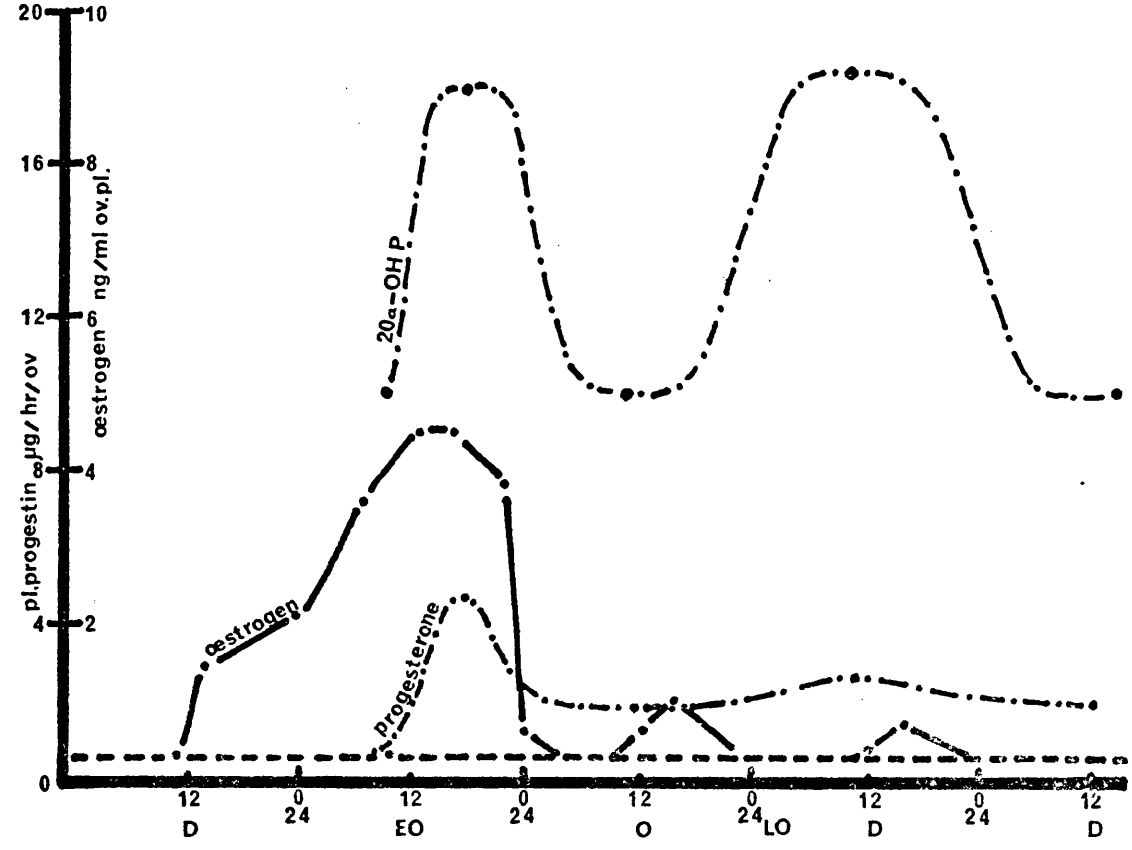
LH levels) and at high titres it prevents LRF secretion. Barraclough & Haller (1970) suggest a possible model where low titres of circulating oestrogen promote both synthesis and release of plasma LH, whilst higher levels gradually inhibit release but not synthesis and finally inhibit both release and synthesis. LH in turn stimulates oestrogen secretion (Gorski). No mechanism *per se* has been proposed for the process of ovulation itself by Gorski, although it is apparently induced by a combination of a daily neural impulse and the appropriate titres of oestrogen and progesterone. A similar mechanism with a daily neural impulse has also been discussed by Holsinger & Everett (1970). Hori *et al* (1968) found a remarkable increase in plasma oestrogen before the critical period of gonadotrophin release.

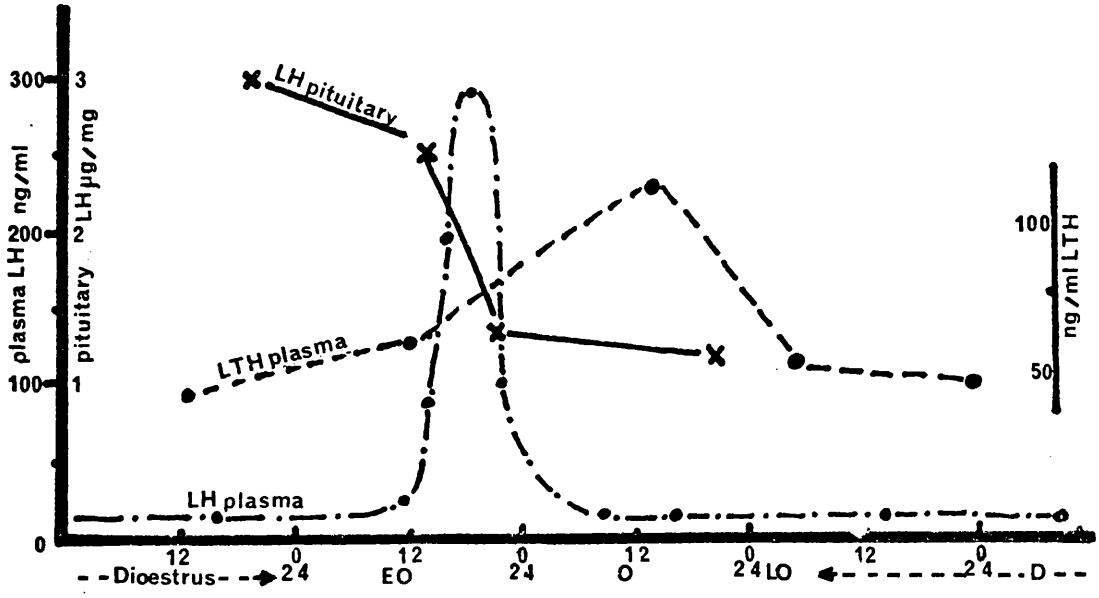
Prolactin has been shown to be luteotropic in the rat by numerous authors, see Bland & Donovan, 1965 and Von Berswordt-Wallrabe & Turner, 1961. LH also has this property and is considered by some to be the dominant tropic hormone (Gorski, 1968; Raj & Moudgal, 1970), prolactin's role being that of preventing the catabolysis of progesterone to the inactive form, 20 α hydroxy-pregn-4-en-3-one (Armstrong *et al*, 1970; Raj & Moudgal, 1970). Progesterone has been noted to rise concurrently with LH (Hashimoto *et al*, 1968), giving further credence to its dependence upon LH, Fig. 5. There is a rise in pituitary follicle stimulating hormone (FSH) from the morning of

FIGURE 5.

Hormonal States During The Oestrous Cycle

FIGURE 5. HORMONAL STATES DURING THE OESTROUS CYCLE





early-oestrus to the morning of oestrus, (Fawke & Brown, 1970). LRF has been shown to be present in hypophysial portal blood of the rat at early oestrus (Fink et al, 1967).

The size of the corpora lutea (trophism) rather than function (tropism) is greatly reduced by hypophysectomy but is only slightly restored by prolactin treatment (Armstrong et al, 1970). They suggested that hypophysial factors other than prolactin are involved in the maintenance of corpora luteal size in pseudopregnancy and growth hormone could be one such factor. Growth hormone (GH) has not yet been shown to vary during the oestrous cycle although increases at ovulation and premenstrually have been reported in humans (Spellacy et al, 1969). GH release in humans can also be stimulated by oestrogen and vasopressin (Frantz & Rabkin, 1965; Gagliardino et al, 1967). Very little is know at present about the regulation of growth hormone although it is thought to be under hypothalamic control. The effects of GH in the rat are better known, increasing protein synthesis and milk production (Greenbaum, 1953; Prosser & Brown, 1962). GH has been shown to be a key factor in the control of aldosterone production (Palmore et al, 1970).

Gestation

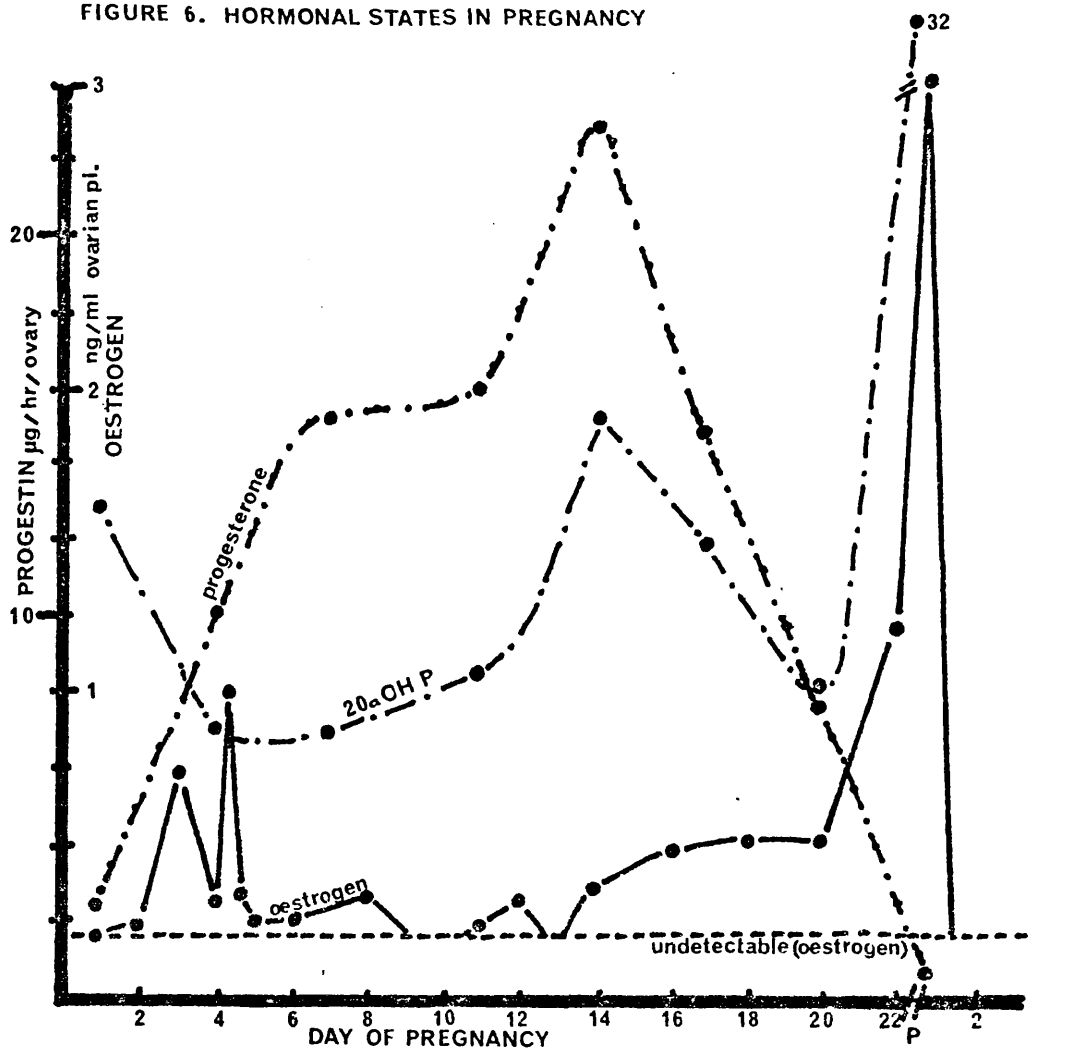
A number of authors have previously suggested that the stimulus of mating triggers a period of luteotropic

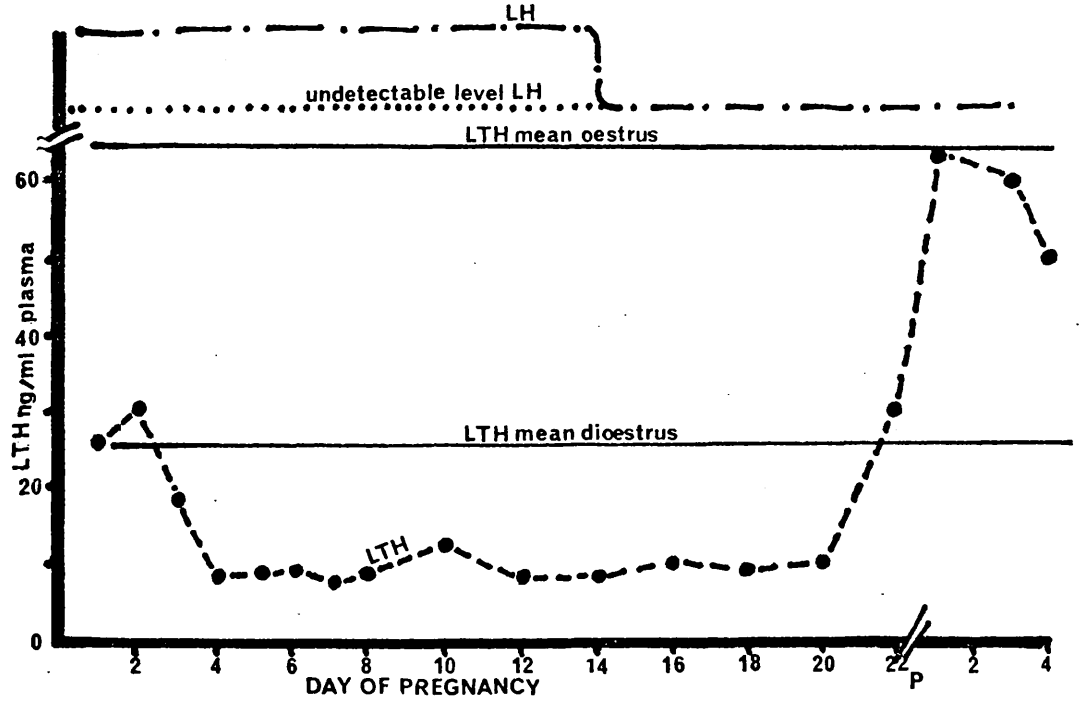
FIGURE 6.

Hormonal States in Pregnancy

(LH indicated merely as detectable/undetectable)

FIGURE 6. HORMONAL STATES IN PREGNANCY





LH could. Deprivation of LH for 2-8 hours or replacement in non-tonic form was found to be injurious to foetal growth during the period up to 12 days. The administration of LH or progesterone during the second half of pregnancy delayed parturition but 20 α OHP (20 α-hydroxypregn-4-en-3-one) and oestradiol had no effect. Resorption of foetuses occurred in rats treated with LH antiserum even when combined with oestradiol replacement therapy, thus suggesting that the basic role of LH is not solely limited to stimulation of oestrogen production. This is in accordance with the findings of Armstrong et al (1970) that both LH and prolactin were necessary for progesterone production, although LH has been found the most active of the two hormones. Pituitary LH increased 3 fold between days 1 and 8 of pregnancy and thereafter remained relatively constant until day 22 (Greenwald, 1966). Pituitary FSH increased 4 fold in the last week of gestation but this was considered to be primarily a storage phenomenon. The secretion of gonadotrophins was believed to remain tonic throughout gestation.

Lyons et al (cited Raj & Moudgal, 1970) reported a prolactin like hormone produced by the rat placenta. Further work by Raj & Moudgal also suggested the presence of an LH like hormone. Csapo & Wiest (1969) have shown that the ovaries themselves become dispensable at some time after mid-pregnancy, the placenta itself providing the necessary hormonal support. Although plasma progesterone levels reflect the general dependence of pregnancy

maintenance on this hormone, it is the uterine titres that are best correlated with maintenance. The theory being that an increase in uterine volume, which is most rapid on day 20, without a concommitant increase in uterine progesterone is a prerequisite for the initiation of labor.

From day 14 there is a fall in plasma progesterone (Csapo & Wiest; Raj & Moudgal) with a decrease in maternal fat stores and increased protein retention (Beaton et al). During this last week of pregnancy there is the greatest period of foetal growth, concurrently there is increased sodium retention (Lichton, 1963). The increased synthesis of protein at the expense of fat stores is a property of growth hormone (GH) (Greenbaum, 1953). As GH has also been shown to be implicated in the control of aldosterone production this could be responsible for the increased sodium retention (Palmore et al, 1970). It is perhaps worth noting that the corpora lutea of pregnancy are not larger than those of the normal cycle until after day 10 (Long & Evans, 1922). Thus whilst LH initiates and maintains corpora lutea for the first 12-14 days of pregnancy and then seems to cease being secreted, the increase in size of the corpora after day 10 may be indicative of the GH activity postulated by Armstrong et al and Beaton et al. Increased pituitary FSH storage in the last week (Greenwald, 1966) may be the factor indirectly responsible for the fall in plasma progesterone. By parturition, 20 α OHP was found to be the predominant progestin in ovarian venous blood

(Hashimoto *et al*, 1968).

The action of GH on the mobilisation of neutral fats involves cholesterol, the precursor of progesterone. LH and prolactin seem to be essentials for this process (Armstrong *et al*) and plasma LH levels are undetectable during the final week of gestation (Midgley *et al*, 1968, cited Raj & Moudgal).

The plasma levels of the principal hormones briefly discussed above are given in Figs. 5 & 6 and are drawn from the data of Yoshinaga *et al* (1969), Hashimoto *et al* (1968), Amenomori *et al* (1970) and Monroe *et al* (1960).

C H A P T E R I I

A REVIEW OF EXPERIMENTAL TECHNIQUES

CHAPTER II

A REVIEW OF EXPERIMENTAL TECHNIQUES

General Conditions of Animal Husbandry

In any study of reproduction in the rat it has been shown essential to take into consideration the effects of the environment.

Light

The effects of various light regimes have been studied (Cooper & Haynes, 1969; Everett & Tejasen, 1967; Dempsey & Searles, 1943) and continuous lighting has been shown (Everett & Tejasen, 1967; Dempsey & Searles, 1943) to induce continuous oestrus by blockage of ovulation, the block being removed by mating.

A number of light regimes have been used: 12 hours of light, 08.00 hrs to 20.00 hrs (Cooper & Haynes, 1969), 07.00 hrs to 19.00 hrs (Dempsey & Searles, 1943); 14 hours of light, 05.00 hrs to 19.00 hrs (Ramirez & McCann, 1964), 07.00 hrs to 21.00 hrs (Fink et al, 1967), 06.00 hrs to 20.00 hrs (Everett & Tejasen, 1967). Luteinising hormone (LH) in rat plasma rises to a peak late in pro-oestrus (Ramirez & McCann, 1964) and a surge of ovulatory gonadotrophins is released between 14.00 and 16.00 hrs in rats subjected to illumination from 05.00 to 19.00 hrs, ovulation occurring some 10 hours later (Cooper & Haynes).

Food

Excessive restriction of food and water has been found to modify the oestrous cycle leading to lengthened and irregular cycles (Cooper & Haynes, 1969), the animals remaining longer in the dioestrous phase with some exhibiting continuous dioestrus. In these dioestral animals normal oestrous cycling has been induced by the use of continuous lighting (Piacsek & Meites, 1965). The irregularities in rats on a restricted diet (70% of unrestricted intake) could also be reduced by feeding just prior to the ovulatory surge (Cooper & Haynes, 1969). Restriction on water intake has been shown to restrict substantially the voluntary food intake and inhibit weight gain (Crampton & Lloyd, 1954).

Temperature

Dempsey & Searles (1943) indicated that heat (95 ± 3°F) and cold (35 to 40°F) had no effect upon the cyclical activity but constant light induced constant oestrous, the temperature ranges were those to be expected in the rat's natural environment. High temperatures (94°F) have been shown to restrict food intake to the extent that the animals lost weight (Squibb et al, 1954) although water was provided ad libitum. Brobeck (1945) found that weight gain was greater at 86°F than at 70°F. Total evaporative water loss in the female rat has been shown to increase sharply above 93°F (Hainsworth, 1968). At 98 ± 2°F and 15-20% r.h. the

rat has been shown to lose 20% of its body weight (as evaporative loss) in eight hours (Horowitz & Borut, 1970). Half of this loss was from the blood plasma and extra vascular volumes which both suffered a 33% loss in volume.

The combined effects of diet, humidity and water restriction on the rate of weight increase of growing male rats has been studied, (Schreiber & Elvehjem, 1954). It was found at moderate temperatures (75 to 79°F) that animals on complete diets and unrestricted water access showed a higher average weight gain at 90% and 60% r.h. (relative humidity), than animals on complete diet with water restriction (2ml/animal/day); further, the water restricted animals at 60% r.h. showed less than half the weight gain of those at 90% r.h. Diets lacking in riboflavine or thiamine fed to rats at 90% r.h. on unrestricted water intake produced similar results to those at 60% r.h. with restricted water intake, the voluntary water consumption of the rats falling.

In newborn Norway rats, the incidence of the necrotic condition of the tail (ring-tail) has been proved to be correlated with temperature and humidity (Totton, 1959), and it is thought to be due to over cooling of the tail caused by excessive evaporation at low temperatures and low humidity. The incidence of ring-tail was completely prevented at temperatures of 68° to 72°F and r.h. $>$ 50% (55% to 60%), (Njaa, Utme & Braekkan, 1957).

It was concluded from the above that in order to conduct

experiments on the normal reproductive physiology of the rat, careful attention must be paid to the lighting regime, the composition of the food, any restrictions imposed on the food and water intake, the time of feeding and the temperature and relative humidity.

Blood Sampling & Handling of Samples

Site of Sampling

There have been a number of places from which serial blood samples have been taken, although frequent bleeding from any one site has been shown to induce anaemia and reticulocytosis (Creskoff et al, 1942). Various cannulation techniques have been employed using the external jugular vein (Kassal & Levitan, 1953) and the aorta (Hashimoto et al, 1968). Heart puncture (Csapo & Wiest, 1969; Burhoe, 1940) and tail vein techniques have also been used (Cushnie, 1954; Rugh & Somogyi, 1969; Newcomer, 1947). Heart puncture and cannulation employed anaesthesia which deeply affects the respiratory and circulatory systems (Westhues & Fritsch, 1964-65) although it has been noted (Hendry, 1962) that anaesthesia has no significant effect upon serum osmolality.

Cushnie (1954) stressed that excessive squeezing of the tail whilst collecting the blood should be avoided and Herrmann (1953) noted that very much higher potassium values were obtained from peripheral blood (humans, finger tip) if the blood drop was squeezed out; sodium and calcium values

were not altered. Slight muscular work i.e. one single clenching of the fist, during the period of sampling from the Cubital vein whilst using a tourniquet, has been shown to lead, in humans, to substantial (up to 1 mEq/litre) and unpredictable increases in plasma potassium concentration (Hultman & Bergström, 1962). It was also found that venous potassium concentration was significantly higher than the potassium concentration of a simultaneously drawn arterial sample.

Time of Sampling

The female rat has been shown to exhibit cyclical peaks of activity in feeding that could be closely correlated to the oestrous phase (Brobeck et al, 1947). In the mouse diurnal variations in activity have been correlated with fluctuations in blood sugar of ± 10% (Bullough, 1949), blood urea levels in the rat were found to increase steadily to a peak at three to four hours after feeding and return to normal by the end of eight to ten hours, the peak level being determined to a large extent by the amount of protein ingested (Parsons, 1930). Glucose loading of the gut leads to dehydration of the body as a whole since it loses more water than electrolytes into the gut lumen and the sugar level in the extracellular fluid is elevated, (Yin, Hamilton & Brobeck, 1970). Sodium chloride solutions produced a similar dehydration.

In humans the importance of pre-sampling diet has been

stressed, patients on high carbohydrate diet may have abnormally elevated glucose levels (Cantarrow & Trumper, 1963). It was advised (Winsten, 1965) to place a patient on a balanced diet for several days prior to the test. Venous, capillary and arterial blood glucose concentrations were nearly the same in fasting humans (Winsten, 1965). After ingestion of glucose by human subjects the concentrations in capillary blood were higher than in venous blood (Reinhold, 1953). Paaby (1959) and Hytten & Leitch (1964) have both indicated the importance of standard conditions being imposed on a subject prior to a sample being obtained and that possible diurnal and seasonal variations should be taken into consideration.

There is evidence from the above that a fasting period prior to sampling would be advisable, the choice of a suitable length and timing of fast would however have to take into consideration the findings (Cooper & Haynes, 1969) that underfeeding can induce a state of constant dioestrus in the rat. It has been suggested (Cooper & Haynes, 1969) that this nutrition induced pituitary block may at least be partially removed and oestrous regularity restored to underfed rats by feeding at 12.00 hrs, the rats being on a 08.00 to 20.00 hrs light regime. The ovulatory gonadotrophin surge (Ramirez & McCann, 1964) occurs at this time and it would be desirable for the maintenance of regular oestrous cycles to begin the period of fasting after this surge.

Pauly & Scheving (1965) pointed out that where diurnal rhythms must be considered, sampling at a given time each day will only lead to consistent results for that particular time and only if the environment is kept rigidly constant. Further, that the conclusions from a comparison of the effects of two different regimes upon a factor can only be valid if a complete cycle of events under each regime has been studied; this is especially important where a factor has its own inherent rhythm. For an example they quote the effects of a light/dark regime and constant illumination (in the rat) upon the circulating eosinophils. By choosing suitable sampling times, but sampling both groups simultaneously, it was possible to show that constant illumination had no effect, increased or decreased the total numbers of eosinophils. Thus it is important to know, when deciding upon a sampling time, if there are diurnal fluctuations and how they are modified by the experimental conditions as opposed to the control. The above complication can be overcome only if samples can be taken at sufficiently close intervals to determine the rhythms, if any.

Handling of Samples & Storage

The collection and preservation of blood samples has been studied both generally (Varley, 1963; Winsten, 1965) and with more specific aims (Funder & Wieth, 1966; 1967 a). Although generally acceptable guide lines may be set out for

the handling of blood samples (Winsten, 1965), the particular method adopted has always been based upon the needs of the following analytical procedure. Thus for the determination of osmolality a mild degree of lysis in the sample would not significantly alter the result, blood cells and plasma having the same osmolality (Hendry, 1962); the potassium content of red cells is however up to 20 times that of the plasma and even a mild degree of lysis would be undesirable. Although, with sufficient sample available it has been shown that corrections can be applied for haemolysis (Mather & Mackie, 1960) it is perhaps best to avoid the problem.

In a sample of blood freshly taken from the animal there was most probably an enzymatic process resulting in a dynamic balance between the potassium in the cell and that in the surrounding plasma (Goodman, Vincent & Rosen, 1954); in the presence of adequate glucose at 37°C the potassium distribution remained steady for many hours. Cooling the blood inhibited the enzymatic process and it was found that there was a greater increase in serum potassium than in blood kept at 25°C.

Sodium redistribution has been found to be the reverse of the above, at temperatures above 0°C the cells have been found to take up sodium in detectable amounts (Beilin, Knight et al, 1966). Storage at room temperatures of whole blood in the presence of large concentrations of heparin (200-300 I.U. per 2-5 ml of blood) has no effect upon the plasma potassium level for more than four hours (Hultman & Bergström,

1962), whilst, with low heparin concentrations, the plasma showed an initial decrease of potassium followed thereafter by an increase (Houtsmuller, 1959).

Loss of carbon dioxide from a sample of whole blood resulted in the passage of chloride from the cell into the serum or plasma (Varley, 1963). The centrifuging of uncapped haematocrit tubes has been shown (Funder &Wieth, 1966 a) to alter the pH of the plasma through carbon dioxide loss; the loss of CO_2 led to a rise of pH of the plasma and water moved from the cells into the plasma (the% of water in the red cells fell). The lowering of the cell water in uncapped tubes was of the order of 1.5 - 3.0 %. Siebert & Rappoport (1955) used an oil to cover the blood whilst contrifuging.

In separated serum, the protein fractions albumin and γ globulin showed a significant decrease after storage for three weeks at -20°C (Gillisen & Groeneveld, 1966). Serum sodium, potassium and chloride have been found not to be affected by storage at room temperature for at least eight hours, in a refrigerator over night (4°C) or in a frozen state (minimum -12°C) for at least one year (Winsten, 1965). Due to glycolysis one half of blood glucose may be lost after two to three hours (Varley, 1963) at room temperature. Cooling to 4°C has been shown to slow this loss down (Goodman et al, 1954), the effect can be controlled by rapid separation of plasma or serum from the red cells and storage in a deep freeze (Winsten, 1965). Osmolality of serum has been found to remain steady over a period of 1-2 hours at

room temperature (20°C) or at 37°C, but if serum is stored over night at 4°C the osmolality falls by 1-2 mOsm/kg (Hendry, 1962).

The Effects of Centrifuging on Plasma Constituents

Early work on the separation of red cells from plasma by centrifuging showed that the packed cell volume could be decreased by increasing both the force applied and the duration of the force (Schlenker & Noll, 1952). It was also observed that the volume of the red cells reached a limiting value and although this value was not the theoretical minimum and there was therefore some trapping of plasma, centrifuging was an acceptable method for determining the relative red cell content of the blood.

The extent of the plasma trapping was studied (Vazquez et al, 1952) using ^{24}Na and albumin ^{131}I markers and it was concluded that the trapping for normal blood could be reduced to less than 2% by prolonged centrifuging at high forces of G. Their estimate of 2% plasma trapping was based upon the albumin ^{131}I figures, the ^{24}Na marker indicated a value of 4% and it was their view that the ^{24}Na diffused into the cells and was therefore an unreliable marker.

Meizels and Remington (1959) showed that of markers used to determine the trapping of plasma, those of large molecular size (e.g. albumin ^{131}I) gave appreciably lower values than those of smaller size (lactose, inulin; Na).

Funder & Wieth (1966 a and b) considered that the term 'trapped plasma' was inappropriate and that the terms 'trapped sodium' or 'trapped albumin' should be used. They concluded that the composition of trapped extracellular fluid differed in composition from the supernatant plasma. Their assumptions were based upon the differential trapping of albumin ^{131}I, ^{22}Na and ^{42}K under conditions in which sodium and potassium fluxes were inhibited. Albumin ^{131}I exhibited 1% trapping, and ^{22}Na 3% trapping; potassium was trapped to the same extent as sodium.

Further work (Funder & Wieth, 1967) confirmed that albumin was trapped to a lesser extent than sodium or potassium, and that the majority of trapped sodium was of definite extracellular origin. The difference in trapping between albumin and sodium remained constant regardless of the packing of the cells or the time employed for separation. It was assumed that the shear of the cell surface was accessible to small molecules and ions but not to large molecules such as albumin.

Analysis

Choice of Containers & Avoidance of Contamination

Observations on the storage of distilled water in hard glass (Pyrex) and soft glass by Dorche & Costet (1955) showed that whilst the water stored in Pyrex containers at

room temperature showed very little increase in sodium content over a period of two years (0.024 mg/litre to 0.23 mg/litre), water stored in soft glass showed a much greater increase in sodium content in six weeks (0.024 to 0.77 mg/litre). Heating to 70°C increased the level of sodium considerably for both Pyrex and soft glass (0.024 to 0.09 mg/litre in 1 hour for Pyrex; 0.024 - 0.12/1.09 mg/litre in soft glass).

The effects of storage on potassium using soft glass and calcium were found to be very slight and of no importance (Dorche & Costet, 1955).

Soft glass can absorb traces of almost any ion or contribute potassium or sodium whilst new glass often exhibits a surface contamination of soluble alkali, most of which can be removed by soaking in distilled water for a few days (Dean, 1960). The treatment of glass surfaces with a silicaon preparation is recommended (Dean, 1960). Experiments conducted by Woldring (1953) on different container materials showed that polythene, or hard glass containers were to be preferred for the storage and preparation of solutions. The use of polythene storage bottles with tight screw caps is recommended (Dean, 1960; Burriel-Marti, 1957), Kautex bottles are also recommended (Zeiss Handbook).

The use of siliconised nylon haematocrit tubes where subsequent electrolyte analyses were carried out has been mentioned (Funder & Wieth, 1966 a).

Many of the surfaces that can come into contact with a

solution can impart traces of contaminants. Finger prints on glassware, and filter paper folded with the fingers can contribute sizeable amounts of sodium (Dean, 1960). Common filter paper may contribute sodium and potassium, whilst acid washed papers strongly absorb elements from trace solutions (Dean, 1960). The grinding of ground glass stoppers in glass bottles will introduce quantities of sodium and potassium into the solution.

Apart from the effects that the loss of water vapour or dissolved gases from the sample or solution may have, the leaving of samples open to the atmosphere can encourage external contamination by particles. Common contaminants present in the air which can affect flame photometric results are brick and cement dust, soap powder and tobacco-ash and smoke (Dean, 1960; Burriel-Marti, 1957). Partial evaporation of volatile organic solvents or even water solutions can produce erroneous results (Dean, 1960).

Serum Osmolality

Osmolality of biological fluids is normally determined from vapour pressure, osmotic pressure or freezing point measurements of the sample. Freezing point methods offer the most simple operation (Johnson & Hoch, 1965) with good accuracy. The freezing point method most commonly used in clinical work is based on determination of the freezing point by the super-cooling of the sample and then utilising the heat released on the instantaneous crystallisation of

water from the sample (Johnson & Hoch, 1965). The method is accurate ($\pm$ 1 mOsm/kg solvent, Johnson & Hoch, 1965; Hendry, 1962), but indirect, requiring correction factors for the effects of super-cooling (Abele, 1933). The method requires samples of the order of 0.2-2.0 ml (Johnson & Hoch, 1965; Abele, 1933).

A more elegant method of determining the freezing point of a solution is to use the thawing point method originated by Drucker & Schreiner (cited Ramsay, 1949) in which the problems of super-cooling are avoided. The freezing point method (Johnson & Hoch, 1965) does however lend itself to automatic methods. The method adopted by Ramsay, (1949) and later modified by Ramsay & Brown, (1955) is a direct method; the sample being initially frozen, is allowed to thaw; the thawing point being synonymous with the freezing point (Ramsay, 1949; Johnson & Hoch, 1965). The method allows measurement of osmolality on samples as small as 2×10^{-5} mm^3 with an accuracy of $\pm$ 1% (Ramsay & Brown, 1955). Ramsay & Brown (1955) pointed out that although the theoretical limit was of the order of 1×10^{-6} mm^3, the method worked best with volumes of 1×10^{-3} to 1×10^{-4} mm^3, the accuracy being of the order of 0.2% at these volumes. Larger volumes, 1×10^{-1} mm^3, were influenced more than the smaller volumes by the effects of latent heat of fusion of ice in delaying equilibrium (i.e. the bath temperature may be well above the freezing point before all the crystals melt.) The presence of protein did not interfere with the determination

of the freezing point (Ramsay, 1949).

Electrolyte Micro-Analysis by Chemical Methods

Shaw, (1954) has made a comprehensive study of the various micro-methods available for the chemical analysis of inorganic ions and has produced a standard procedure which was both accurate and simple. Shaw's technique was drawn from the work of Wigglesworth (1938) on chloride; Linderstrøm-Lang, Palmer & Holter (1938) on chloride; Cunningham, Kirk & Brooks on potassium (1941); Norberg (1937) on potassium; Lindner & Kirk (1937) on calcium; and Sobel & Sobel (1939) on calcium (cited Shaw, 1954). Shaw has added to their work the estimation of magnesium (see table 6).

The procedure (Shaw, 1954) was the same for each ion. The body fluid sample was first dried in an oven at 100°C and then ashed at 450°C to remove organic material. A suitable precipitating agent was added (see table 7) to the ashed sample, the precipitate was separated by centrifuging, washed and dried. The precipitate was then converted to the chloride and titrated with the aid of a simple but very effective micro-burette, the end point being determined either with an indicator or electrometrically. The conversion of the ion to the chloride allowed the final determination to be carried out using a single titration solution of silver nitrate.

Recovery of the chloride for calcium, magnesium and potassium was very good (Table 7) but for sodium the recovery

Table (6) - Micro-Analysis for Electrolytes (from Shaw, 1954)

ION	Precipitation Agent	Titration Method	Author	Sensitivity & Accuracy
Cl^-	Silver nitrate	Excess silver nitrate with sodium thiocyanate	Wigglesworth, 1938	-
Cl^-	-	Direct with silver nitrate using electrical end point	Linderstrom-Lang, Palmer & Holter, 1935; Cunningham, Kirk & Brooks, 1941a; Ramsay, Brown & Croghan, 1955, 1st method	4µg Cl^- ± 4.5% 5µg Cl^- ± 2.0% 1µg Cl^- ± 1.0%
Cl^-	-	Direct titration with silver ions using electrical end points plus capacitor charge	Ramsay, Brown & Croghan, 1955, 2nd method	10^{-4} µg Cl^- or 0.5×10^3 µl to ± 1.0% SD
Na^+	Zinc uranyl acetate	Reduce uranyl ion and titrate with ceric sulphate	Lindner & Kirk, 1938	-
K^+	Chloro-platinic acid	Reduced with sodium formate and titrated with silver nitrate	Cunningham, Kirk & Brooks, 1941b	-
K^+	Chloro-platinic acid	Converted to iodoplatinate and titrated with $Na_2S_2O_3$	Norberg, 1937	-
Ca^{++}	NH_4 oxalate	Excess ceric sulphate and back titrated with ferrous ammonium sulphate	Lindner & Kirk, 1937	-
Ca^{++}	NH_4 oxalate	Converted to calcium carbonate and titrated with acid	Sobel & Sobel, 1939	-

Table (7) - Micro-Analysis of Calcium, Magnesium, Potassium & Sodium

ION	Precipitating Agent	Precipitate	Conversion to the soluable chloride	Recovery from single solutions of salts	Body fluids accuracy for 1µg of ion
Calcium	5% soln. ammonium-oxalate at pH5 to prevent co-precipitation of magnesium	Calcium oxalate	calcium oxalate (+ heat) → calcium carbonate, (+hydrochloric solution) ⟶ calcium chloride	100%	± 0.01 µg
Magnesium	5% 8 hydroxy-quinoline (on the supernatant from calcium determination to avoid calcium interference)	magnesium-organic complex	magnesium organic complex(+ heat)→ magnesium oxide, (+ hydrochloric acid) ⟶ magnesium chloride	100%	± 0.01 µg
Potassium	4% chloroplatinic acid in 80% ethanol	potassium chloro-platinate	potassium chloro-platinate, (+formic acid + heat) ⟶ potassium chloride	100%	± 0.01 µg
Sodium	saturated zinc uranyl acetate	sodium zinc uranyl acetate	sodium zinc uranyl acetate, (+ammonium nitrate + heat)→ sodium zinc uranyl oxide, (+hydrogen chloride gas)⟶ sodium zinc uranyl oxy-chloride	82% - 83% (constant) chloride correction was applied	± 0.04 µg

From the data of Shaw, 1954

was a constant 82% to 83% and a correction factor had to be applied. Mutual interference of magnesium and calcium was of a low order (Shaw, 1954). The larger error for sodium determinations (± 4%) arose during the process of separating the precipitate of zinc uranyl acetate from the supernatant and it was suggested that the accuracy might be improved by adoption of a filtering technique rather than centrifuging (Shaw, 1954). The method (Shaw, 1954) allowed determination of potassium, calcium and magnesium at the 1 µg level with an accuracy of ± 1%.

The use of the electrometric end point by Ramsay et al, (1955) allowed the determination of the chloride content of biological fluids to be carried out on samples ranging from 0.2 to 10 µl in volume and from 10 to 500 mEq/litre in chloride concentration: the standard deviation was quoted as less than ± 1%. Protein did not interfere with the potentiometric end point and there was no error due to the presence of phosphate, sulphate, oxalate, citrate or benzene sulphonate (Ramsay et al, 1955).

In a second method (Ramsay et al, 1955) the titration of the chloride was accomplished by the direct liberation of silver ions. A current was passed through the sample solution using a silver electrode in series with a condenser; silver ions were liberated and the charge developed on the condenser was a measure of the chloride titrated. The method dealt with volumes down to 0.5×10^{-3} µl and could measure 10^{-4} µg of chloride with an error of ± 1% (SD). The authors

(Ramsay et al, 1955) believed the condenser charge to be an absolute measure of the amount of chloride titrated.

Electrolyte Analysis by Flame Spectrophotometry

The possibility of using both atomic absorbtion & emission spectrophotometry for quantitative as well as qualitative analysis has a long history (Prugger) but it is only during the last few decades that they have been developed to their present state of reliability. The subject of flame photometry and its clinical application is well covered by Dean (1960), Burriel-Marti and Ramirez-munoz, (1957), McIntyre (1961) and Ramirez-Munoz (1968).

Atomic absorbtion methods in which the attenuation of a suitable background radiation by the dispersed atoms is measured, have only become acceptable since 1955 (Walsh, 1955) but offer a useful complementary to the longer established emission methods. Both methods are applicable to serum electrolyte analysis but for sodium, potassium and calcium the emission method is preferred because of its greater sensitivity. The analysis of magnesium, which has a very high excitation potential and is not easily measured except in very hot flames, is best carried out using atomic absorption methods where the flame is used merely to disperse the atoms.

Micro-analysis of sodium and potassium by emission

Emission flame photometry has been used by a number of authors (Table 8) to analyse the sodium and potassium content of very

Table (8) - Micro and Ultrmicro Analysis for Serum and Plasma Sodium & Potassium

SAMPLE	ELEMENT	METHOD	DILUENT	SAMPLE SIZE	DILUTION	ACCURACY & SENSITIVITY	AUTHOR
-	Sodium	integrating flame photometer emission	direct	1 µl	zero	3% to 8%, 0.05 to 2.0% in pure solution	Ramsay, (1950)
serum	Sodium (589.0mµ 589.6mµ) and Potassium (766.5mµ 769.9mµ)	Emission	aqueous (+5% TCA)	500 µl	1:10	± 2%	Smit, Alkemade & Verschure, (1951)
		Filters	also various alcohols and water	-	-	gave 6-8 increase in emission	
-	Sodium & Potassium	integrating flame photometer emission	direct	10^{-3}µl	zero	at lower limit of 4 x 10^{-8}mg Na^+= 17% but larger amounts of Na^+ and K^+ ± 3%	Ramsay, Brown & Falloon, (1953)
serum	Sodium & Potassium	flame emission	aqueous diluent	50µl for the 2 together	1:10	± 1% to 2% for Na^+ & K^+	Hermann & Baumann, (1953)
serum	Sodium & Potassium	flame emission	aqueous diluent	5 µl	1:100	± 2.3% potassium ± 1.0% sodium	Hermann, (1953)
serum	Sodium - 590 mµ Potassium- 770 mµ	flame emission monochromator with total consumption	organic diluent acetone/acetic acid/water	20µl for the 2 together	1:500	reported 100% recovery of added elements. lower detectable limits of dilution. Sodium x 200, 000 Potassium x 2,000	Kingsley & Shaffert, (1954)
-	Potassium 770 mµ	Filter emission	organic diluent 20% methanol in water plus sodium	200 µl	1:50	± 0.3%	Siebert & Rapoport, (1955)
plasma	Potassium	Emission	aqueous diluent	100 µl	1:20	SD ± 0.33 at 3.76 mEq/ litre, 5% low in aqueous solution (constant), used correction factor	Hultman & Bergström (1962)
serum and plasma	Sodium & Potassium	Emission and special "Unopette" capillary system	aqueous plus lithium	13 µl	1:200	Sodium ± 1.4% Potassium ± 2.2%	Walker & Gerrade, (1966)
serum and plasma	Sodium & Potassium	emission monochromator spray chamber or total consumption	aqueous, compensated diluent and wetting agent	Na^+ 5 µl K^+ 40 µl	Na^+ 1:400 K^+ 1:50	± 1% for both	Zeiss, (1968)

small volumes of biological fluids such as blood serum and plasma.

The sample size has varied from 500 µl (0.5 ml) for determination of sodium and potassium (Smit et al, 1951) to 10^{-3} µl (Ramsay, Brown & Falloon, 1953). Dilution of the sample has varied from nil (Ramsay et al, 1953) to 1 : 500 (Kingsley & Schaffert, 1954).

The work of Smit et al (1951) showed that small samples could be analysed with reasonable accuracy ($\pm$ 2%) using conventional equipment. They reported that dilution of the serum with distilled water (1 : 10) gave low values for sodium and potassium (- 20% and - 30% respectively) and that prior precipitation of the protein with tri chloroacetic acid prevented this. They concluded that this was due to the precipitation of α andβ globulins which took a percentage of the serum alkalies with them. They allowed for the mutual cationic interference of sodium and potassium by calculation from a series of graphs prepared from data collected by atomising standard solutions of sodium at varying potassium content and vice versa: as potassium in small quantities had very little effect upon the sodium content, the sodium content was determined first and the appropriate graph of potassium x emission at that sodium concentration was then consulted for the potassium content. No attempt was made to produce a straight line graph for sodium concentration x emission

although it was suggested that the addition of a caesium salt would strengthen the emission curve for sodium and prevent the variable mutual interference of sodium and potassium. The use of organic solvents was investigated and was found to give up to 8 x enhancement of emission but it was not adopted for the main method.

The emission changes due to the viscosity differences of sample solution and standards noticed by Smit et al (1951) were eliminated by the use of wetting agents e.g. sterox (Kingsley & Shaffert, 1954; Dean, 1960; Burriel-Marti, 1957; Zeiss Handbook) and by great dilution (Walker & Gerrade, 1966; Dean, 1960; Burriel-Marti, 1957).

The 30% loss of potassium in 1 : 10 water diluted samples reported by Smit et al (1951) was found by Hultman & Bergström (1962) to be 5% when compared with trichloro-acetic acid diluted samples when using a dilution of 1 : 20 with water, furthermore, this was found to be constant whatever the concentration of the protein. It was therefore considered possible to use an empirical correction factor of 5% for the potassium loss due to protein precipitation.

Siebert & Rapoport (1955b) added sodium to the diluent for potassium analysis to ensure an excess of sodium. The addition of an excess of sodium or caesium for potassium analysis was also recommended by Dean (1960) and Burriel-Marti (1957). In their work on the influence of sodium, calcium, barium, lithium and methanol upon serum potassium emission, Siebert & Rapoport (1955a) showed that sodium

and methanol have a definite enhancing effect that could be utilised to increase the sensitivity of emission methods. They also showed that the enhancement effect of sodium reached a definite maximum and that the error in potassium evaluations due to serum sodium variations could be overcome by the addition of an excess of sodium to the sample. This enhancement effect and its asymptotic nature was also reported by Smit et al (1951). Siebert & Rapoport (1955a) found that diluting the serum 1 : 50 with a diluent containing 4.5 mEq Na/litre of 20% methanol in water raised the emission for potassium about two times, and gave clear solutions with cat or dog serum. With human serum a slight turbidity appeared which did not effect the potassium values i.e. there was no precipitation loss as mentioned by Smit et al (1951) and Hultman & Bergström (1962). This turbidity, which increased with alcohol concentrations, could be removed by addition of acetic acid or sodium hydroxide.

The use of various alcohols and other organic solvents to raise the emission of serum sodium, potassium and calcium, was studied extensively by Kingsley & Shaffert (1954) who concluded that the optimum diluent was a mixture consisting of 67.5% acetone, 22.5% glacial acetic acid, 9.98% water and 0.02% sterox (a wetting agent). The acetone raised the emission whilst the water-acetic acid portion prevented the loss, by protein precipitation, of serum alkalis, particularly calcium. Sterox was primarily added to prevent adsorption of calcium ions on to the walls of the glass containers,

but it also reduced the surface tension of the solution and promoted better droplet formation. The use of this diluent solution allowed a dilution of 1:500 to be used, and sodium, potassium and calcium to be analysed from 20 µl of serum. They reported a recovery of added elements of 100%.

The action of alcohols and surface active agents (wetting agents) e.g. sterox, in promoting a greater emission is poorly known. Siebert & Rappoport (1955a) noticed that consumption rate decreased with increasing alcohol content and that whilst under the same physical conditions sodium emission was increased 1.3 fold, calcium emission was increased 2.0 fold and potassium 2.5 fold. They concluded that whilst the increase was due partially to the better atomisation of the solutions, there was a further effect of the alcohol.

Kingsley & Shaffert (1954) noted that when uptake rate of the various solvents was corrected to that of acetone, the fastest feeding, all except diethyl-ether were relatively equal in their enhancement effect on sodium, potassium and calcium: i.e. it was the physical properties that caused the primary increase. Dean (1960) has noted that sterox alone could increase the emission by presumably promoting better droplet formation, larger drops tending to both cool down the flame and to be trapped in the antechamber (in non total consumption burners). Dean has suggested that the enhancing effects

of alcohols etc. were due to a change in flame dimensions, the increased nebulosity of the spray (very important with condensing chamber burners) and the decreased cooling effect on the flame due to the larger water droplets.

The problem of evaporative loss errors incurred in using the acetic acid/acetone/water mixture of Kingsley & Shaffert (1954) could be avoided by the use of an isopropanol/water mixture (60:40) containing 0.02% sterox, without much loss in sensitivity or protein precipitation (Dean, 1960).

The method recommended by the manufacturers (Zeiss, 1968) for the Zeiss PMQII series flame photometer was one in which a common diluent solution was used for both the preparation of the standards from a single principal calibration solution (containing sodium, potassium, calcium and magnesium). In this way, as long as both principal calibration solution and sample were diluted in exactly the same ratios, precise measurements of volume could be avoided. The diluent solution, consisting of N/10 hydrochloric acid plus 0.02% wetting agent, was added directly to the serum without prior deproteinisation. The acid prevented precipitation of calcium as the carbonate and the opalescence due to partial precipitation of the proteins.*

---

* Hydrochloric acid had no effect on emission spectra of sodium, potassium and calcium (Kinsley & Shaffert, 1954). Proteins are less soluble in the pH range of 5.2 - 5.4 (the isoelectric point) also with increasing organic solvent concentrations (Mahler & Cordes, 1968).

Standard deviation for sodium and potassium determination amounted to less than 1% and interference by sodium with potassium emission less than 0.4% at the limits of the normal physiological range.

Vaginal Smear Technique

In 1889 Morau observed a cyclic change in the vaginal epithelia of rats and mice which was independent of pregnancy, the length of this cycle of events was given as ten days (cited Long & Evans, 1922). Work on the guineapig by Stockard and Papanicolou (cited Long & Evans, 1922) enabled them, by inspection of the vagina and by microscopic examination of the smears, to determine the time of oestrus and ovulation, and the length of the cycle.

Long & Evans in their classical monograph on the oestrous cycle in the rat showed that the length of the cycle in the rat was about four days and that it is characterised by regular, periodic and coordinated histological changes in every portion of the genital tract, especially the epithelia of vagina and uterus.

Classification of cycle phases

Long & Evans divided the cycle into five distinct phases, of two main divisions: the oestrum, and the dioestrum or resting stage. They based their division of the cycle by vaginal smear on the two premises i) that each of the recognisable stages was found to be approximately constant between animals in contrast to other

phenomena, ii) that the vaginal smears constituted the only reliable method of recognising the sub-divisions of the oestrous cycle in the intact living animal. Their scheme and the correlation of vaginal smear to the changes in the reproductive organs is given in table 9.

Emery & Schwabe (1936) expressed doubt that the cycle could be so sharply divided into stages as suggested by Long & Evans. Astwood (1939) in work upon the changes in uterine weight during the oestrous cycle, divided the cycle into six phases (table 9). Astwood's divisions were to some extent dictated by the need to predict the appearance of the pro - oestrous (stage I of Long & Evans) phase and its conclusion. He found that the confirmatory and typical nucleated epithelial cells of pro-oestrus were preceeded by the appearance of loosely formed groups of small oval and rounded cells of varying size admixed with a few leucocytes. This phase he named pre-oestrus.

In work based on the classifications of Astwood (1939) and Hartman (1944) Mandl (1951) divided the cycle again into six phases but re-classified the nomenclature: the pre-oestrous phase of Astwood becoming the late dioestrous phase, in accordance with general ideas of Long & Evans that the onset of the oestrum was heralded by the sudden cessation of leucocytic migration through the mucosa.

Mandl's classification (1951) was at some variance with previous work regarding the metoestrous (stage IV) phase of Astwood (1939) and Long & Evans. Long & Evans

TABLE (9) - COMPARISONS OF THE SUBDIVISIONS OF THE OESTRUS CYCLE OF ASTWOOD AND MANDL WITH THOSE OF LONG & EVANS

LONG & EVANS (1922) 110 hrs	STAGE I PO (12) Non acceptance of male. Increasing turgescence of vaginal folds. Vaginal mucosa opaque. Sudden absence of leucocytes. Sheets of neucleated cells. N.EP.		STAGE II EO——(27)—— Heat exhibited. Swelling of vaginal lips. MATING. Sudden replacement of neucleated epithelials by the cornified cells. No leucocytes. C.	STAGE III LO Non acceptance of male. Swelling may persist. Ovulation occurs in this phase. Greater accumulation of cornified cells. Cheesy mass in vagina near cervix. Vagina dry. OVULATION. C.	STAGE IV MO (6) Swelling of vaginal lips disappearing. Corns disappearing. Sudden appearance of leucocytes. Reappearance of neucleated epithelials. Cheesy mass becoming fluid. C.,N.EP.,L.	STAGE V or DIOESTRUM (48-53) No swelling of vaginal lips. Mucus in lumen of vagina. Mucosa moist. Many leucocytes. Free epithelial cells (single) Polymorph leucocytes. Few or no corns. C.,L.	
ASTWOOD (1939) 109 hrs	PO (4) As Long & Evans	PO/EO (3) Neucleated and corns (50/50)	EO——(25)—— As Long & Evans	LO As Long & Evans	MO (8) As Long & Evans	D (55) As Long & Evans	PPO (4) A few leucocytes and loose groups of small, rounded cells of varying size. Sparse smear.
MANDL (1951) 107 hrs	EO (18) Thick smear with large numbers of hexagonal basophilic neucleated epithelials. Honey comb pattern sheets. Absence of leucocytes. N.EP.		O (early)—(25)—— Epithelial cells have separated, lost their nuclei and have cornified. Smear becomes cheesy late in oestrus. No leucocytes. C.	O (late) C.	LO (5) Appearance of basophilic epithelial cells with corns, also Shorr cells. C,N.EP.	ED (24) MO is now part of ED Sudden appearance of leucocytes, LO merges with ED on appearance of leucocytes. Some large Shorr cells. Some small nucleated basophilic epithelials. Thick smear, almost entirely leucocytes. Some corns. L.,V.EP.,C.,S.,N.EP.	D (28) Thin smear consisting of leucocytes, vacuolated basophilic epithelial cells, few cornified, few Shorr, few Hartman type V cells. L.,V.EP.,C.,S.,H.	LD (7) Few leucocytes, vacuolated and also neucleated basophilic epithelials. No corns or Shorr cells

stated that the inauguration of stage IV was the appearance of leucocytes among the cornified cells of oestrus and that during this phase there was a reappearance of the nucleated epithelial cell of pro-oestrus or stage I. Mandl correlated her phase of late oestrus (of similar duration) with the metoestrous (stage IV) phase of Astwood and Long & Evans, but her definition of the phase was based on the reappearance of nucleated epithelial cells amongst the cornified cells before the appearance of leucocytes. The leucocytes indicated the rapid transition into early dioestrus.

Length of Cycle

Apart from the work of Morau (cited Long & Evans) based on sections of the vaginal epithelium, most workers have given a period of four or five days as the length of average oestrous cycle in the rat.

Long & Evans reported a mean length of 113 hours with a mode of 93, the major difference between the two being in the duration of the oestrous phase (38 hours and 27 hours respectively). Astwood (1939) using a very large number of 1 x daily smears, rather than the three-hourly smearing of Long & Evans, found a mean of 109 hours. Mandl (1951) using the method of Astwood, gave a mean of 107 hours and concluded that the elongated oestrous phase of the Long & Evans study was due to too frequent smearing, the results including some animals in continuous eostrus. This conclusion was borne out by the observations of

Emery & Schwabe (1936) and Ball (1937) that incorrect or too frequent smearing induced lengthened oestrous phases.

The variance in cycle length was found to be significantly less within than between individuals, and litter mates tended to show cycles of a more similar length than unrelated animals (Mandl). It was further shown by Mandl that the variance in cycle length of the dioestrous phase, the oestrous phase remaining more or less constant for all animals.

The time of Ovulation in Relation to the Smear

Ovulation occurred during the last hours of the cornified cell stage (stages II & III) and always by the end of stage III, or by the appearance of the first leucocytes in the vaginal smear (Long & Evans). Ova could be observed in the distal fold of the oviduct between 18 and 30 hours after the appearance of the first cornified cells i.e. the beginning of stage II, but not before, and Long & Evans concluded that ovulation normally took place during this period. Cooper & Haynes (1969) placed ovulation at the beginning of this period, 10 hours after an ovulatory surge of LH (c. 0200 hrs. on the day of oestrus - Fig 1.)

Staining Techniques

The collection of the debris from the lumen of the vagina has varied (table 10) both in frequency with which

Table (10) - Vaginal Smear Techniques

AUTHOR	AVERAGE CYCLE LENGTH	FREQUENCY OF SMEAR TAKING	METHOD OF SMEAR TAKING	FIXATIVE	STAINING TECHNIQUE
LONG & EVANS (1929)	4.6 days 110 hrs.	1 x daily and 1 x 3 hr.	speculum spatula	-	observed unstained in Ringer's or Locke's fluid
EMERY & SCHWABE (1936)	-	2 x daily 4 x daily	saline lavage and cotton swab	-	observed unstained in saline
ASTWOOD (1939)	109 hrs.	1 x daily	-	-	-
HARTMAN	4 days	-	cotton swab	wet fix in 50:50 ether, 95% ethanol	Schorr (1941) trichrome. Harris haematoxylin/Biebrich scarlet - Orange G/Fast green F.C.F. (modified Massons trichrome)
EVERETT (1948)	4 and 5 days	1 x daily	saline lavage	dry fix in alcohol	1% alkaline toluidine blue
MANDL	4.4 days 107 hrs.	1 x daily	wire loop	modified schandinn solution 50:50 ethanol/saturated mercuric chloride	1. rapid:- Mann's eosin blue 2. detailed:- 1% ponceau-de-xylidine red/2-5% methylene blue

it has been collected and the device with which the debris have been removed. Long & Evans (1922) used the medical method of speculum and spatula and collected samples for the detailed cycle study once every three hours. This has been criticised by others (Mandl, 1951; Astwood, 1939) as possibly inducing lengthened oestrous phases. Emery & Schwabe (1936) concluded that whilst frequent swabbing (4 x daily) with a cotton swab frequently induced oestrus-like smears, the saline lavage seldom produced this effect and that a reduction in frequency to 2 x daily with lavage was advantageous. It was found that an oestrus-like smear could be induced in the castrated female if frequent swabbing was used. Ball (1937) suggested that prolonged cornification was a symptom of disturbed endocrine balance rather than a local response to irritation as indicated by the fact that the condition could be correlated with decreased sexual excitability, some animals being more susceptible than others.

The use of a wire loop to obtain 1 x daily samples by Mandl (1951) did not apparently interfere with cycle regularity.

Preparation of the smear prior to observation has varied considerably (table 10). Shorr (1941) and Hartman (1944) have pointed out that the drying out of cells prior to fixing alters both the morphology and the staining properties of the cells and should therefore be avoided. Mandl (1951) did not state whether the smear was fixed in a wet or dry state and Everett (1948) dried the smears prior to fixing

in alcohol.

A number of authors did not stain the smears before observation; of those who did, two gave single stains for rapid examination; Everett (1948) used 1% alkaline toluidine blue and Mandl (1951) Mann's eosin blue. To allow a more complete differentiation Hartman (1944) used a trichrome staining method derived from an earlier method of Shorr (1940). This method gave excellent results but was considered too lengthy for routine work and a single differential stain was described (Shorr, 1941) allowing a sufficient degree of differentiation without lengthy preparation.

The single differential stain (Shorr, 1941) provided sharp differentiation between cornified and non cornified elements and clearly differentiated leucocytes, erythrocytes, bacteria and spermatozoa.

Mandl (1951) in a second method, used Ponceau-de-xylidene red dye, counter-stained with methylene blue. Differentiation was not as clear as with the trichrome stains of Hartman (1944) or Schorr (1940) but was more rapid and differentiated between basophil and acidophil cytoplasm.

Cell types present in the vaginal smear and correlation with oestrous phase

Long & Evans (1929) were concerned solely with three cell types in determining the oestrous phase from the vaginal smear; the leucocyte, the nucleated epithelial cell and the cornified cell (Table 9).

Table (11) - EPITHELIAL CELL TYPES

From the data of Hartman (1944)
Stain = Shorr trichrome (1940)

Hartman Cell Type	Common Name	Colour	Nucleus	Cytoplasm	Notes
I	Scales	brilliant orange red	none or ghosts only	cells clear and glassy, usually devoid of granulation. 1st scales plastic, later ones rigid and less opaque	The cell at full oestrus
II	Shorr cell	blue to blue/green cytoplasm	full round or slightly elliptical, granular, chromatic, i.e. vesicular	large, clear	as large as or larger than scales. Origin, beneath the stratum corneum
III	Basophils				
1.	The basophilic Shorr cell	blue but may be chemically transitional to the red cornified condition	as above	contains basophilic granules	originate from the stratum granulosum
2.	the 'late' basophil	light green cell	vesicular nucleus	few scattered basophilic granules, roundish cytoplasm	first epithelial cells to appear in EO after the leucocytes disappear. Astwood's pre-oestrous cell?
IV	Mucous cells	(variable)	-	vacuolised, scattered at first, later full blown mucous cells	usually found on the third (dioestrous) day
V	Phagocytosed cell	purple	-	contains leucocytes (may be surrounded by a halo)	various epithelial cells staining purple, containing one or more leucocytes, contemporaries of mucous cells
VI	Other Epithelial cells				
Class 1.	Dense cells	red-purple	full	-	appear in early metoestrum (LO/ED) in company with leucocytes
Class 2.	Deep cells	red-purple to light green later	pyknotic	-	degenerating cells but appearing after denudation at day 4 i.e. dioestrus
Class 3.	Red epithelial	red	full and finely granular red	dense and finely granular	considered a precornified cell from upper layer of stratum corneum. Many cells will appear thus if allowed to dry
Class 4.	True Prooestrus	green to blue	grey nuclei uniform distribution of chromatin	(clear)	Large cell appear at disappearance of leucocytes. Differ from Shorr cells in the nucleur chromatin distribution
Class 5.	See late basophil (Type III 2)				
Class 6.	Degenerating cell	light green	dark (black) pyknotic elliptical to crescentic	cells edges indistinct, subtly vacuolated	cervical in origin, accompanies leucocytes and cervical mucous (light green) around D and LD

Table (12) - Smear Picture & Oestrous Phase

PHASE	DESCRIPTION
Early Dioestrus	Thick smear, consisting almost entirely of leucocytes: some cornified cells, Shorr cells and a few basophilic epithelial cells.
Dioestrus	Thin smear, consisting of leucocytes, basophilic epithelial cells undergoing vacuolation, practically no cornified cells, diminishing numbers of Shorr cells, and Hartman's Type V.
Late Dioestrus	Leucocytes, some vacuolated and some clearly nucleated; basophilic epithelial cells, no cornified cells, no Shorr cells.
Early Oestrus	Thick smear consisting of large numbers of hexagonal basophilic nucleated epithelial cells, often disposed in a honeycomb pattern. Towards the end of this phase, the cells separate and cornification sets in.
Oestrus	All, or almost all, the epithelial cells have lost their nuclei and have become cornified. Towards the end of this phase, the smear becomes 'cheesy'.
Late Oestrus	The smear is similar to the oestrous smear, but, in addition to many cornified cells there are come large basophilic Shorr cells and some small basophilic epithelial cells. As soon as the leucocytes appear, late oestrus merges into early dioestrus.

NOTE: Smears were often diagnosed as being intermediate between two of the above stages (e.g. LD/EO, LO/ED, etc.)

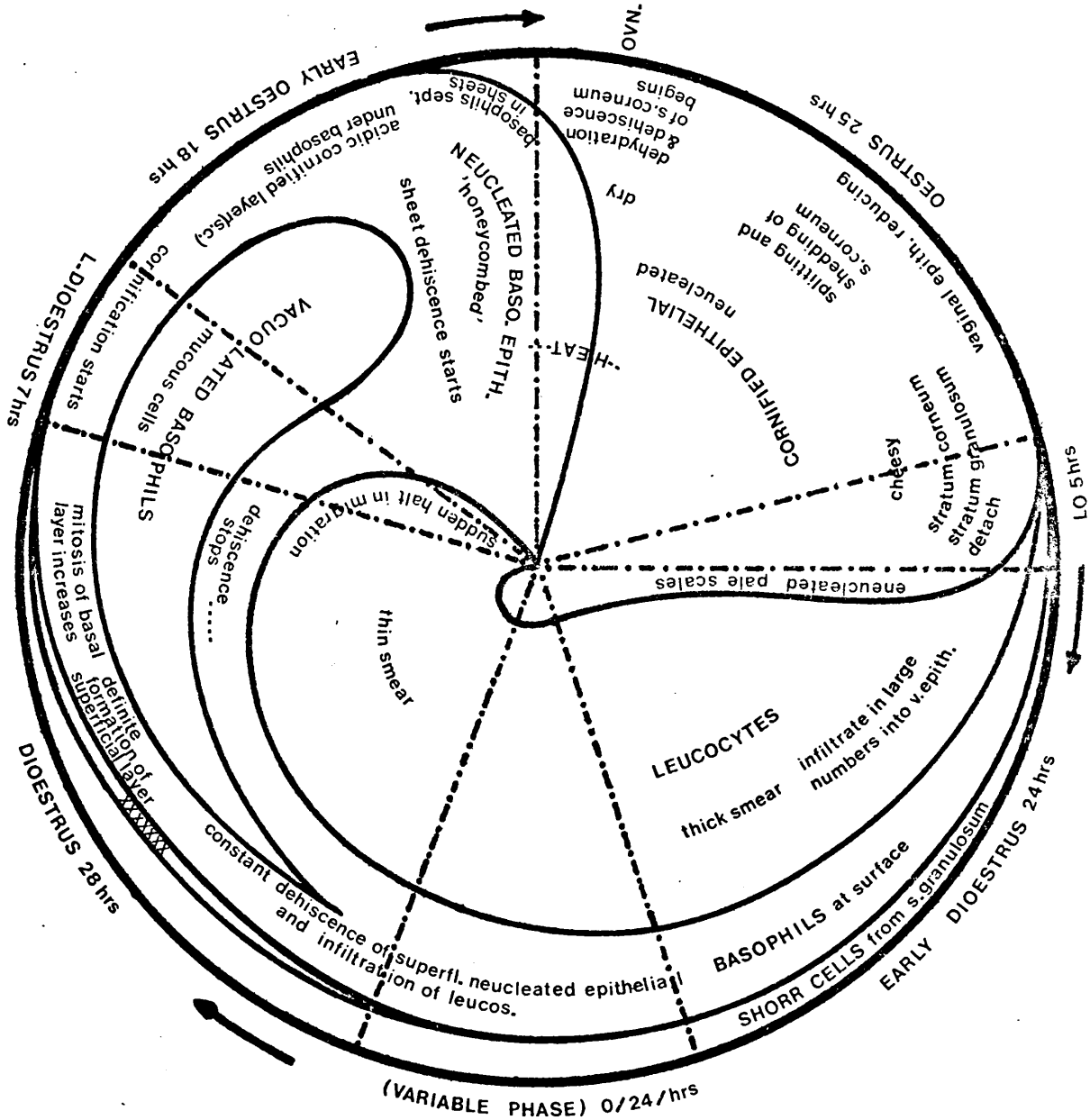
From data of Mandl (1951)

FIGURE 7.

Composition of Vaginal Smear

During the Oestrous Cycle

FIGURE 7. COMPOSITION OF VAGINAL SMEAR DURING THE OESTROUS CYCLE.



xxxx Hartman's type 5 cells

Hartman (1944) using the trichrome staining method of Shorr (1940), with slight modifications, found advantageous for rat smears, produced a most comprehensive classification of the various epithelial cell types of the rat vaginal smear (table 11). In most cases the probable origin was given as well as the time of appearance in the cycle. Astwood's (1939) pre-oestrous cells (table 9) were described and named the 'late basophil' (cell type III 2, table 11) but its origin was unknown; it did however differ considerably from the true pro-oestrous epithelial cells.

Mandl (1951) used a simplified classification based on the work of Astwood (1939) and Hartman (1944) to produce a diagnostic description of the expected smear picture at each of the six main phases of her revised cycle (table 12).

A comprehensive collation of the information provided by the various authors mentioned above has been presented in Fig. 7.

Accuracy

Mandl (1951) in a systematic check on the accuracy of the diagnosis of vaginal smears showed that the mean error of diagnosis was 0.3 stage per reading, and that at no time was there a disagreement with a previous diagnosis of more than $1\frac{1}{2}$ stages. With continuous reference to previous diagnoses the mean error would probably have been smaller.

C H A P T E R I I I

MATERIALS AND METHODS

CHAPTER III

MATERIALS AND METHODS

Animal Husbandry

The Animals

The animals were derived from a closely inbred albino strain: "Harvey" strain, Aberdeen University Physiology Animal House. These were crossed with the Stirling Animal House commercial strain of albinos to give a vigourous but easily handled strain. The experimental animals were chosen randomly from this stock, the only factors determining the choice being similar age (but > 10 weeks) and similar weight (c. 200 gms).

The animals were individually housed in wire topped plastic cages on expanded mica (vermiculite). The cages were arranged so that there was an approximately similar amount of illumination incident on each cage, the uppermost layer were shielded by a shelf. The males were kept near the females to encourage normal cyclic regularity. The animals were handled daily to accustom them to the experimenter. Animals were not used in experiments until they had exhibited at least two oestrous cycles of normal length (4 to 5 days). Immature animals normally ovulate for the first time at approximately 76 days of age and the first cycles are usually abnormally long (c. 10 days), (Long & Evans, 1920)

Environment

The temperature and humidity of the animal room were kept as constant as the facilities allowed. The room heating and ventilation were controlled electronically with the temperature at 75°F ± 2°F throughout the year, occasional extremes of external temperature were not controllable but these periods did not coincide with an experimental period. Humidity was controlled to a certain degree by keeping the floor of the room moist; the relative humidity varied between 50% and 75% throughout the year. The temperature and humidity were recorded continuously on a thermohydrograph calibrated by the wet and dry bulb method (Met. Office pamphlet). These conditions were the same as those imposed on the general breeding stock to prevent ring tail and promote good weight gain.

Light regime

The lighting of the animal house as well as the experimental room was controlled by time switch to give a light period from 06.00 hrs to 20.00 hrs i.e. 14 hrs of light. There was no other source of light. The animals used in the experiments were thus adapted to the light regime from birth.

Food

The food fed to the animals was Oxoid rat breeding diet, which contained extra vitamin A and D_3. Except where indicated the food was fed ad libitum; similarly

the water was available at all times except where indicated. The importance of an adequate water and food supply in maintaining full breeding vigour and cycle regularity has already been indicated.

Mating

Two males were caged with each female prior to the onset of oestrus (in late dioestrus) and removed after twelve days. Two males allowed for infertility in one of the males and twelve days covered at least two complete oestrous cycles. At the end of twelve days most females which had mated showed significant weight increases.

Sampling and Sample Handling

Time of sampling and duration of pre-sampling fast

The lack of specific information on pre-sampling fasting in experiments involving the rat meant the evolvment of a suitable regime. There were three regimes used, each of which will be described and briefly discussed. All animals were on a 06.00 hrs to 20.00 hrs light regime.

REGIME I - (a group of unmated females, blood sampled daily and smeared daily)

These animals were allowed free access to food and water from 14.00 hrs to 01.00 hrs, the food was removed at 01.00 hrs and the water at 09.00 hrs. Sampling was

conducted from 13.00 hrs to 14.00 hrs, giving a pre-sampling fasting period of 12 hrs and water deprivation of 4 to 5 hrs. This regime was started one week before commencing to sample.

This regime appeared to be too harsh, some animals lost condition, exhibiting the red pigment on head and body indicative of stress conditions (Shreiber & Elvejem, 1954). Net weight gain was very poor and some animals showed a net loss of 10 gms over a period of 3 weeks. There was no sign of anaemia and previous work (Maybank, 1966) indicated that sampling at 1 day and 2 day intervals did not induce stress.

It was concluded that under the existing conditions of husbandry a fasting period of twelve hours induced a state of stress in the animal.

REGIME II - (a group of females, mated during the experiments, blood sampled daily and smeared daily)

The fasting period was reduced to seven hours, with free access to food and water from immediately after individual sampling, c. 17.00 hrs to 09.00 hrs when food and water were removed simultaneously. Sampling was conducted from 16.00 hrs onwards and took approximately $1\frac{1}{2}$ hrs.

The results of shortening the fasting period to seven hours were mixed; although there was no incidence of red pigmentation and the oestrous cycles generally appeared

regular prior to mating, there was a poor body weight gain even during gestation and the mating and littering performance was poor (average number per dam 7.3, average litter weight 44.8 gms). Two animals exhibited constant dioestrus.

The appearance of constant dioestrus in two animals and poor overall mating performance indicated that the fasting conditions were the cause of the poor results (Cooper & Haynes, 1969). These animals were probably more susceptible than others to food and water removal (Cooper & Haynes, 1969; Hainsworth, 1968; Hainsworth, Sticker & Epstein, 1968).

REGIME III - (a group of mated and unmated females, sampled every three days and weighed at food removal)

The animals had free access to food and water after sampling (18.30 hrs to 19.30 hrs) until 13.00 hrs when food and water were both removed. This gave a fasting period of $5\frac{1}{2}$ to $6\frac{1}{2}$ hrs commencing one hour after the critical time for food removal, 12.00 hrs (Cooper & Haynes, 1969). Free access to food and water was given on non-sampling days.

The adoption of a six hour fasting period was based on the results of a short pilot experiment upon the effects of food and water removal on plasma potassium and sodium and serum osmolality. The fasting period of six hours was inserted between the critical time of Cooper & Haynes (1969)

(12.00 to 13.00 hrs) and the beginning of the dark period at 20.00 hrs.

No signs of stress were observed and mating performance was very good; 8 out of 10 females mated and became pregnant within two days of introducing the males. The litters were all above average in numbers and of good vigour. The controls showed signs of the regular cyclical weight changes correlated with oestrous phase (Brobeck et al, 1947).

The improvment in performance by the animals under this sampling/fasting regime may have been due to the better synchronization of fast and hormonal state of the animals but firm conclusions cannot be drawn because of the lesser strain imposed by sampling at three day intervals. To span the whole of pregnancy with 10 samples it was necessary to space the sampling at three day intervals.

Site of Sampling

The tail tip method of Cushnie (1954) was used. The animal was imobilised in an adjustable crush and the tail completely immersed for one/half minute in a warm dilute solution of Dettol (45°C, 5ml/litre). The tail was swabbed with absolute alcohol then xylol, wiped with a clean tissue and the tip laid on a firm wad of clean filter paper. A small slice (c. 1mm) of the tip was removed with a clean scalpel and the first drop of blood discarded. The blood (c. 70µl) was then taken up into the appropriate

centrifuge capillaries by capillary action (plain for osmolality; heparinised for electrolyte analysis).

A small amount of liquid paraffin (reagent grade) was introduced in the tube above the sample to prevent CO_2 and water loss (Varley, 1963; Funder & Weith, 1966a). The unwetted end was plugged with Cristaseal (Hawksley). Care was taken to prevent trapping air in the column of fluid. Replicate samples were taken where there was sufficient blood.

Very gentle squeezing of the tail helped the blood flow but strong squeezing was avoided. With practice between 60 µl and 100 µl could be withdrawn. After sampling, the tail was wiped with a clean tissue and dipped into an antiseptic solution of 0.1% acriflavine. Ten or more samples were taken at daily intervals with no evidence of anaemia.

Subsequent Handling of Samples & Storage

The samples in the capillary tubes were kept at room temperature in a vertical position to preserve boundaries, prior to separation of the red cell column, (necessary to prevent potassium flow from the cells to the plasma, Goodman et al, 1954; Hultman & Bergström, 1962). Net sodium transfer was kept to a minimum by rapid separation of the plasma from the cells (10 to 15 minutes).

The blood samples were centrifuged in a micro haematocrit centrifuge (Hawksley & Sons, Lancing, Sussex)

at 12,000 G for five minutes. The haematocrit values were measured and the tubes were scored, cleaned and the red cells, with the buffy coat, and 1 mm of plasma were discarded. The plasma or serum was subsequently blown out into the appropriate storage tubes and stored at -17°C until required for analysis.

For freezing point the samples were stored under paraffin in o.5 ml polythene sample tubes.

For electrolytes the samples were initially stored without paraffin in cut down polythene tubes with tight stoppers. The approximate volume of the storage tube was 0.1 to 0.2 ml. Occasionally it was found that samples gave very high electrolyte readings, possibly due to evaporation of the plasma water where the stopper was not a good fit.

For experiments III and IV the samples were kept in their glass centrifuge capillaries with a small volume of paraffin above and below the sample. The samples were frozen as soon as possible in a portable cold box and stored overnight at -17°C for analysis the following day. The samples were warmed rapidly in the hand and left for five minutes just prior to dilution. Storage in the glass tubes for more than two weeks gave erratic electrolyte values.

Haematocrits and the Use of Lithium Heparin

Without heparin there was no separation of white cells from red cells in the small bore tubes and the boundary

between cells and serum was indistinct. The haematocrit values were between 1% and 2% higher than those found when heparin was used and the convex boundary was difficult to read. Heparinised tubes gave sharp divisions between supernatant plasma, buffy coat and red cells and the haematocrit values were more reproducible.

Heparin is usually obtained as the sodium or potassium salt which will interfere with sodium or potassium analysis. The tubes were therefore changed with 10 I.U. per tube of pure lithium heparin (Evans Medical Limited, Speke, Liverpool). The specifications of lithium heparin were:-

sulphated ash	not more than 35%
content of lithium	3.95%
content of potassium	negligible ($<$ 0.01%)
content of sodium	0.2 - 1.0%
content of calcium	less than 0.03%

Heparinised tubes could not be used for freezing point analysis because of the high electrolyte content. A short experiment indicated that values 20% higher than the average could be expected if heparinised tubes were used for freezing points.

Haematocrits were measured with a micro-haematocrit reader (Hawksley).

Analysis

Containers and the Cleaning of Glassware

Storage of solutions Calibration solutions for both osmolality and electrolyte determinations were stored in screw-top polythene bottles under a dust cover. The solutions were well shaken and the exterior portion of the thread neck wiped with a clean tissue to remove any dried fluid before dispensing the contents. All beakers of fluid were kept covered with a watch glass (or parafilm in the case of alcohol diluents).

Where glass containers were used at room temperature they were of pyrex, for hot fluids vitreosil crucibles were used.

Cleaning of containers Plastic containers were cleaned with hot and cold deionised water, care being taken to avoid contact with the inner surface by the fingers. Containers were dried in an air oven at 70-80°C under tissue for 12 hours, and stored in dust proof containers.

All glass and vitreosil (beakers, haematocrit capillaries, freezing point glassware) was cleaned by prolonged immersion (12-24 hrs) in fresh chromosulphuric acid to remove organic residues and grease. This was followed by prolonged washing in hot tap and deionised water, soaked for 24 hours in cold deionised water and a further 10 rinses in cold deionised water. The glass was then dried

for 12 hrs under tissue in an oven at 110°C and stored in dust free containers.

Measurement flasks and pipettes were treated as above but were not allowed to come into contact with hot fluids, nor were they dried in an oven. To prevent contamination from the fingers, clean vinyl gloves were worn whilst washing glassware.

The Measurement of Serum Osmolality

Serum osmolality was determined from the measurement of the thawing point(freezing point) using the ultra-micro method of Ramsay & Brown (1955). A number of modifications were made to the apparatus, ancillaries and method to give greater ease of operation and improved accuracy.

The cooling chamber The apparatus followed the basic design of Ramsay & Brown (1955) but greater attention was paid to the insulation of the freezing bath from sudden fluctuations in ambient temperature.

The freezing bath was separated from the heater controls and light source both for better heat insulation and convenience . The air space between the perspex freezing bath and the containing box was filled with a two inch layer of expanded polystyrene, the outer walls and floor of the box being constructed of $\frac{3}{4}$" plywood with a 'formica' facing. The 1" thick perspex lid of the box was manufactured to close tolerances to reduce air convection

and all joints and apertures were sealed. The metal parts in contact with both the bath and the exterior of the apparatus were heavily lagged with expanded polystyrene.

The inner cooling vessel was replaced by the cooling probe of a portable freon refrigerator unit (Type TK1 of the Rhenische Geratebau GMBH, Buchs SG, Switzerland). This model provided a maximum cooling rate of 500 Kcal/hour and was fitted with an adjustable valve for regulation of cooling rate. In normal use the box could be cooled from room temperature to -0.600°C in two hours; for very rapid cooling the valve could be fully opened to give working temperatures in half to three quarters of an hour.

Heating rate was controlled by two high amperage rheostats, giving a coarse and fine adjustment, with the voltage across the heater element monitored by a meter. This simple arrangement gave a continuously variable heat output of adequate stability. For greater stability and control the assembly could be replaced by a variable output constant voltage unit. The heater circuit was arranged so that a single three position switch could control cooling, fast heating and slow heating.

The samples of serum were taken up into the vitreosil capillary in the same manner as in Ramsay & Brown (1955) but the capillaries were thoroughly rinsed with boiling deionised water and dried over hot asbestos immediately prior to use. The mounting of the sample capillary and

and its protecting tube on the specimen carriage differed slightly from Ramsay & Brown (1955). Instead of mounting the sample and its tube with sealing wax, it was slid into a short length of plastic tubing fized horizontally to the carriage. To prevent the leakage of alcohol into the protecting tube, the end of the tube was sealed with putty (Cristaseal).

Prior to immersion in the freezing bath the sample was frozen by spraying with an aerosol cooling spray (Radiospares freezer), care being taken not to shatter the sample by too rapid cooling.

The light source was an adjustable bench light with focussing and an iris diaphram. The bench light was fitted with a heat filter and had provision for coloured filters. With suitable angleing of the light beam, adjustment of focus and iris, and the use of a blue filter, the ice crystals in the sample could be made to stand out well from the rest of the sample.

Measurement of Temperature The temperature of the coolant in the freezing chamber was measured with an integrating quartz thermometer - Hewlett Packard HP 2801A, using an HP 2850A probe. The quartz thermometer had a direct digital display of the temperature and mode, and was linked to a digital print-out for continual monitoring of the temperature. The maximum temperature resolution was 0.0001°C, the minimum rate of reading at this resolution

being 10 seconds.

The probe was the smallest in the range with a thermal heat capacity of 0.5 gm of water and a volume of c. 1.5 ml. The cable from the probe to the instrument was constructed from Teflon and of negligible thermal leak rate (1×10^{-3} cal/sec/°C.)

The principle of the quartz thermometer is the sensitivity of the resonant frequency of a quartz crystal to temperature change. The main advantage of the quartz thermometer is the good linear frequency/temperature relationship. Although of very high resolution, linearity and stability, the quartz thermometer must be periodically calibrated in this application to compensate for zero-drift.

Characteristics of the quartz thermometer:-

The validity of utilising the quartz thermometer in this application was based partly on the data provided by the manufacturers and partly on observations drawn from trials. Although with sufficient precautions a mercury glass thermometer could have provided comparable absolute measurements of temperature, the greater resolution of the quartz thermometer made it a suitable choice for what was essentially a comparative study. A further advantage was the automatic recording of the temperature at precise time intervals, essential for the adjustment of the temperature rise to a given rate.

The characteristics of the quartz thermometer

influencing its accuracy were as follows:-

Linearity of probe readings

- The extent to which the digital display may be in error due to non linearity of the temperature/ frequency relationship.

Hysteresis effects

- The extent to which readings differ from calibrated values due to the frequency offset incurred after a temperature excursion.

Response time of probe

- The percentage of the final value reached in a given time, equivalent to "lag" in a mercury in glass thermometer.

Sample rate

- The error in temperature readings due to the time factor involved in the frequency counting.

Stability

- Short term stability or reading to reading variation (= standard deviation) due to internal noise of instrument.

- Zero drift

- Ambient temperature effects

The values of the above in the present application are given in the Appendix.

The two factors having the greatest influence on the repeatability of the instrument were the error due to ambient temperature changes and zero drift. Zero drift was checked by measuring the freezing point of a standard solution but during the analysis of over 100 samples there was no measurable drift. Error due to ambient temperature changes could have been reduced to $< 0.001°C$ by the immersion of the sensor oscillator in an ice bath. The changes in room temperature were however slight and had little apparent effect upon the instrument.

Calibration of the thermometer The thermometer was calibrated to the ice point ($0.0000°C \pm 0.0003°C$) using an ice bath of crushed ice and water made from deionised water distilled from permanganate (Hewlett-Packard application note 78-1 Precise temperature measurements using the quartz thermometer).

The probe and dewar flask were throughly cleaned prior to calibration. The ice point was corrected for barometric pressure.

After the zero setting of the instrument, the freezing point of three standard calibration solutions were measured as an additional check. The solutions

were prepared according to the data of Johnston & Hoch (1965 - see note in Appendix). Repeatability was tested on a single sample and upon different samples.

Micro-analysis of Blood Plasma for Sodium & Potassium

A number of authors have recommended that flame photometric analysis should be carried out on blood serum rather than plasma because of the larger protein content of the plasma. If plasma is to be used then the proteins should be precipitated with trichloro-acetic acid (T.C.A.) first (Dean, 1960; Burriel Marti, 1957; MacIntyre, 1961). Not all authors have agreed about the effect of plasma or serum proteins upon sodium and potassium values (Smit et al, 1951; Hultman & Bergström, 1962; Siebert & Rappoport, 1955b). It was decided to avoid the complications of using T.C.A. on the very small samples available in this study, bearing in mind that the sodium or potassium loss, if any, is constant and calculable (Hultman & Bergström, 1962).

The use of lithium heparin as an anticoagulant to prevent lysis and to give a good haematocrit result, necessitated the use of plasma for flame analysis.

The aims of the following methods were to simplify the repetitive procedure whilst providing maximum protection against interferences. In the case of

potassium it was found necessary to work at the limit of sensitivity of the flame photometer.

Interferences MacIntyre (1961) divided these into the following categories (each factor will be briefly explained):-

i) Spectral Interference

- the interference due to the close proximity or overlap of emission lines (or bands) to the element under analysis. Sodium has suitable strongly emitting lines at 589.0 mμ and 589.5 mμ (MacIntyre, 1961). A suitable slit width (e.g. 0.05 mm) gave good separation (effective band width passed by the Zeiss M4QIII monochromator at this slit width was 3.2 mμ).

Potassium was analysed at 768.0 mμ and 0.14 mm slit width. This gave an effective band width of 20 mμ and covered the strongly emitting wavelength doublet at 766.5 mμ and 769.9 mμ without interference from the sodium lines at 818 mμ and 819 mμ. The emission of radiation by the flame itself provides a continuous spectrum or bands and depends upon the gases used. Hydrogen/air provided the best

metal to background signal with a weak continuous spectrum in the 300 mμ to 400 mμ region (Dean, 1960).

ii) Cationic Interference (Cationic Enhancement)

In a flame a certain percentage of ground state atoms of an element will ionise and thus be unavailable for emission (decaying ions emit different wavelengths to those emitted by an excited atom returning to the ground state - MacIntyre, 1961.

Where two elements are in solution together they will both contribute to the ion content of the flame and as the ionisation process can be considered as a dissociation process with an equilibrium constant (MacIntyre, 1961) they will thus mutually increase each other's percentage of atoms in the ground state, leading to increased emission. The degree of ionisation depends upon the ionisation potential of the element and the flame temperature (Dean, 1960).

Due to the very low ionisation potentials of both sodium and potassium (5.12 ev and 4.32 ev respectively), there will be

mutual enhancement where these two elements occur in solution together.

Variations in the concentration of sodium will lead to fluctuations in the degree of enhancement and vice versa. The enhancement effect is asymptotic (Siebert & Rappoport, 1955b) and if there is an excess of interferent present there will no longer be an error due to its fluctuations.

To overcome the mutual interference of sodium and potassium an excess of caesium chloride (ionisation potential 3.87 ev) was added to the diluent solution, this had the three fold effect of increasing the emission of sodium and potassium whilst supressing any fluctuations due to variable enhancement and strengthening the concave emission curve for potassium at low concentrations. A cool hydrogen air flame was used to reduce interference and flame background radiation was also reduced.

iii) Anionic Interference

- Due to the formation of compounds of certain metals with certain anions which vaporize with difficulty, this was not of importance for sodium and potassium.

iv) Self Absorption

- The absorption by atoms in the ground state in the cooler, outer zone of the flame of emissions by atoms returning to the ground state in the inner hotter part of the flame (absorption and emission can occur at the same wavelengths - resonance lines). This effect is normally found above certain limiting concentrations giving a convex curve e.g. sodium is especially prone to this effect but not seriously, with magnesium it is a major problem. Closely spaced calibration solutions were used to determine the calibration curve for sodium; potassium at these concentrations had a linear concentration/emission relationship.

v) Spray Interference

- Variation in emission due to the different physical properties of the compared solutions e.g. temperature, surface tension, viscosity and boiling point (Dean, 1960). The use of high dilutions and wetting agents are two of the methods available to overcome this effect which is important with serum or plasma analysis.

The plasma samples were diluted 1:400 for

sodium and 1:200 for potassium and the diluent contained 0.02% of a non-ionic wetting agent.

Readings for both elements were read after 20 seconds to allow for the haze from the spray to pass into the flame at a constant rate and to eliminate any remnants of the previous solution. Solutions kept in a refrigerator overnight were allowed to reach room temperature before analysis. Solutions were stored in a refrigerator if not used within a few hours.

The Instrument The instrument used for analysis was the Zeiss PMQII series flame spectrophotometer consisting of a burner with spray chamber (FAII) single quartz prism monochromator (M4QIII) and a detector unit with two photometric detectors for measurements in the visible, ultra violet and infra red regions, a photomultiplier for 185-700 mμ and a PbS photo conductive cell for 600-2500 mμ. The detector unit was coupled to a Servoscribe (Smiths Industries) 20 cm single channel pen recorder, with a 5,000 MFD condenser across the input as a 7 sec. damper.

Optimum Working Conditions of the Flame Spectrophotometer The best settings of air flow, gas flow, slit width and amplification were found for each element

and diluent type. For sodium these settings were not critical but potassium analysis was conducted at the limit of detection of the instrument and in this case the settings were most important. Dean (1960) suggests that it is a wise precaution to use a lean flame i.e. slightly more air than fuel gas and to work at the settings which give maximum emission. For potassium the optimum settings were found by a series of trials, plotting emission against one variable factor whilst keeping the other factors constant, see Figs. 8, 9 & 10. For aqueous solutions the air flow reached a maximum level. For iso-propanol solutions this did not occur, Fig. 8. This was presumably due to the cooling effect of the water solution upon the flame and the fact that the air flow could not be increased sufficiently in the case of the alcohol for it to reach its maximum value for emission. In the case of variable hydrogen flow with iso-propanol the effect of a lower air flow can be seen, Fig. 9, in (a) the maximum aspiration rate of the sample has not been reached.

The effects of increasing the slit width, Fig. 10, were first to increase the galvanometer deflection and then to decrease it - this was due to the necessity of setting the galvanometer to zero for each amplification. Above 0.15 mm the background signal was too great for the electrical zeroing circuit.

FIGURE 8.

Effects of Air Flow and Alcohol on

Emission

FIGURE 8. EFFECTS OF AIR FLOW AND ALCOHOL ON EMISSION

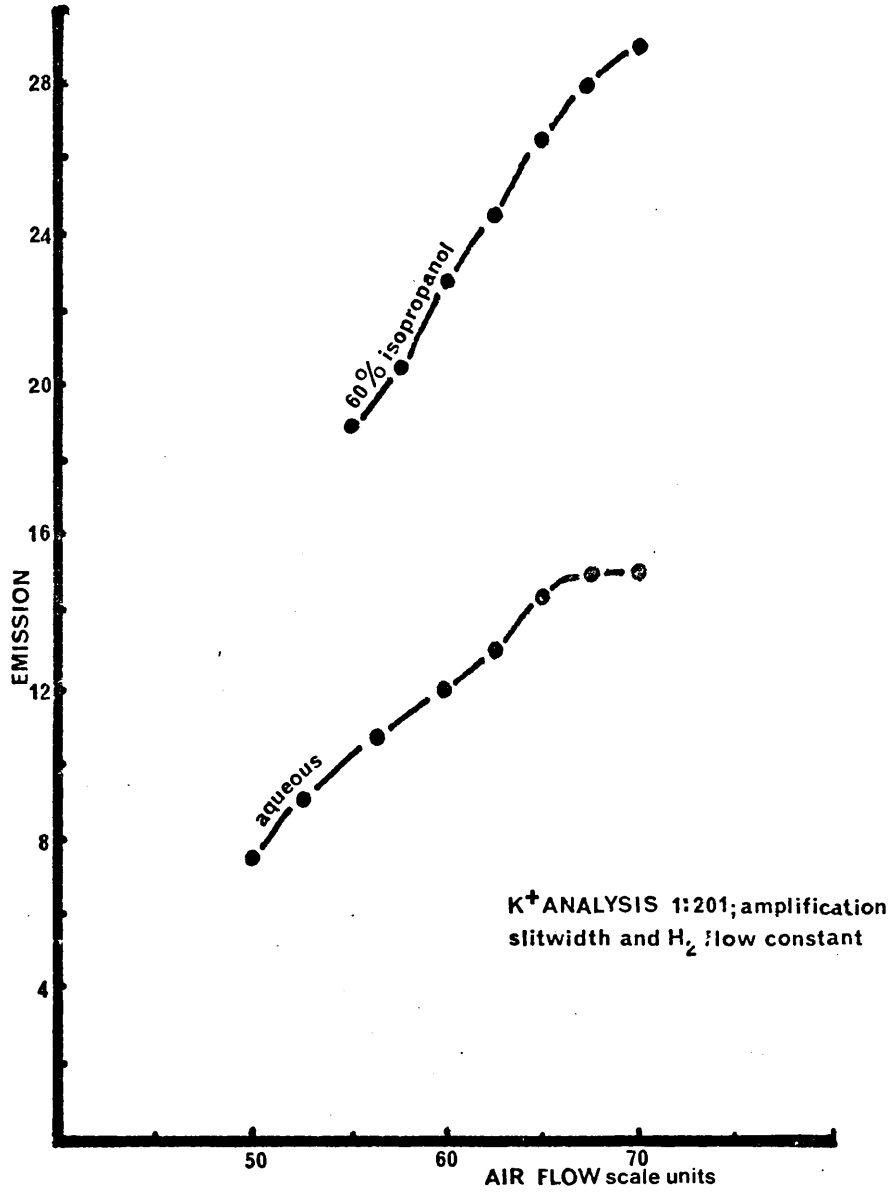


FIGURE 9.

Effects of H_2 Pressure on Emission

FIGURE 9 EFFECTS OF H_2 PRESSURE ON EMISSION

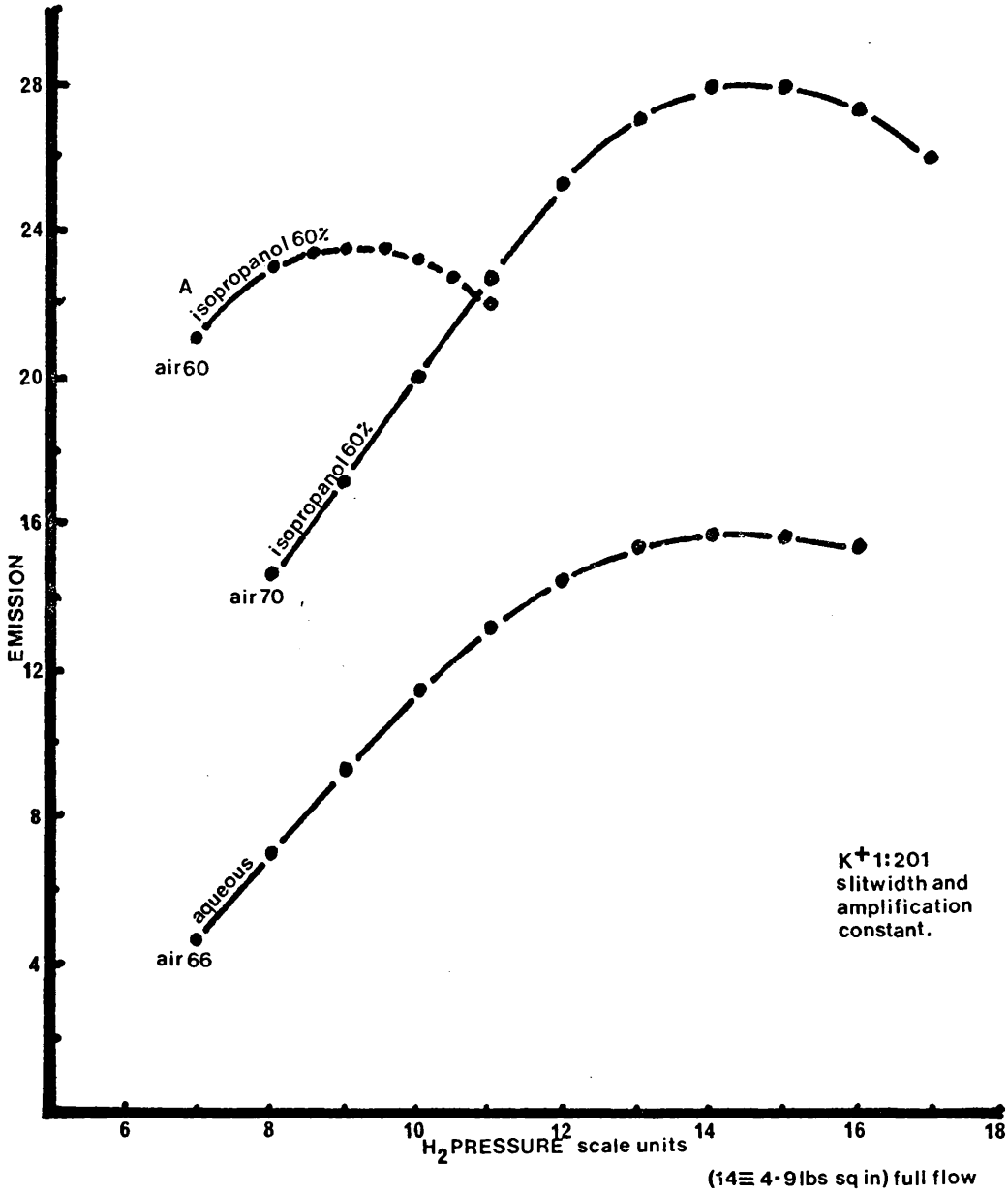
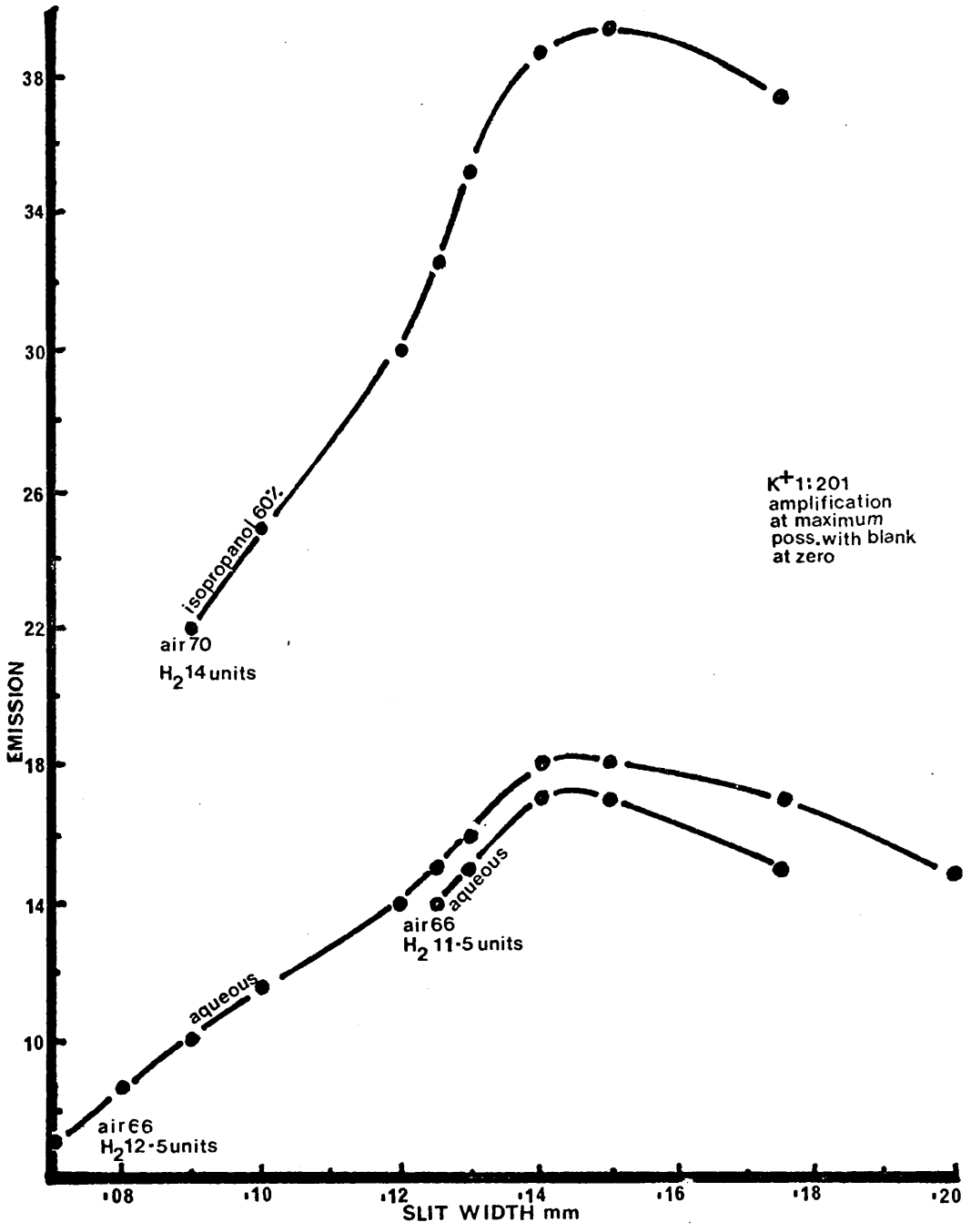


FIGURE 10.

Effects of Slit Width and Alcohol on Emission

FIGURE 10 EFFECTS OF SLIT WIDTH AND ALCOHOL ON EMISSION



Hermann's Method - After Zeiss The modified method of Hermann (cited Zeiss) utilised an aqueous principal calibration solution (PCS) containing the following cations in the concentrations normally found in human serum:

Na^+	K^+	Ca^{++}	Mg^{++}	
330	15	10	2	mg/100 ml
143	3.85	4.99	1.64	mEq/litre

The above solution and the sample of serum were diluted in precisely the same ratios with a common diluent solution consisting of N/10 hydrochloric acid containing 0.02% non-ionic wetting agent. The diluent solution was used as a blank solution to determine the background emission of the diluent and the flame. The method was recommended for serum analysis without prior precipitation of the proteins.

The addition of a common diluent in equal ratios to both serum and principal calibration solutions avoided the complex volumetric measurements of other methods. The addition of 1 gm/litre of a caesium salt to the diluent was recommended to compensate for fluctuations in sodium and potassium.

Enhancement of Emission The analysis of potassium in 5 µl of plasma indicated a dilution of 1:200 (Maybank, 1970). Although this dilution gave the plasma sample comparable physical characteristics to the calibration solutions, there was a corresponding loss in emission that could not be made up by the amplification reserve of the instrument.

The maximum reading of net deflection above flame and solution background was 17 scale divisions which did not provide sufficient sensitivity, Fig. 10. The use of a 60% v.v. solution of iso-propanol (propan-2-ol) in the preparation of the diluent increased the net deflection to 40 scale divisions and reduced the sample aspiration rate by over one half that of the aqueous solution.

It was found necessary to keep all solutions containing iso-propanol well covered to prevent a significant change in values due to evaporation.

Sodium Analysis The samples were analysed in a hydrogen air flame using the instructions in the Zeiss handbook for sodium serum analysis at 1:401. The principal calibration and diluent solutions were prepared from ampoules ("Titrisole" Nos. 9976 and 9979 respectively, E Merck, Darmstadt, West Germany) and 1 gm/litre of reagent grade caesium chloride was added to the diluent. The solutions were made up with

deionised water in class A volumetric flasks and stored in screw topped polythene bottles.

Dilution Procedure:-

1. 2 ml of caesium diluent was pipetted with a class A bulb pipette into a 10 ml pyrex micro beaker.

2. 5 µl of lithium heparinised plasma was added to the 2 ml of caesium diluent using a 25 µl micro syringe with Chaney adaptor (Hamilton series 700, Micromeasure, N.V., The Hague, Netherlands). The beaker was covered with parafilm and the contents well shaken.

3. The calibration solution was made up in the same manner as above using PCS instead of plasma.

2 ml of solution gave 2 x 20 sec readings. The same pipette was used for both standard and plasma diluent measurements and the same two divisions on the Hamilton syringe were used to measure the 5 µl of plasma and PCS. Between samples the syringe was cleaned with deionised water and dried in a syringe cleaner.

Potassium Analysis The samples were analysed in a hydrogen/airflame at the optimum flow rates for air and hydrogen found in preliminary trials. The wavelength used was 768 mµ at a slit width of 0.14 mm, the red sensitive detector was used.

The sample was diluted 1:201 (5 µl of plasma in 1 ml of diluent) using a 60% v.v. propan-2-ol/aqueous

diluent (see Appendix) containing 1 gm/litre of caesium chloride.

Dilution Procedure:-

1. 1 ml of propan-2-ol diluent was pipetted with a class A bulb pipette into a 10 ml pyrex micro beaker and covered with parafilm.

2. 5 µl of lithium heparinised plasma was added to the 1 ml of diluent using a dry 25 µl Hamilton syringe, the beaker was re-covered and the contents well shaken.

3. The calibration solution was made up in the same way using principal calibration solution instead of plasma.

The syringe was cleaned with deionised water between samples and dried in a syringe cleaner. 1 ml of solution gave two twenty second readings. The diluted samples were stored in a refrigerator if not used within a few hours.

Both sodium and potassium sample solutions were prepared consecutively from the one sample of plasma. The syringe was filled with say 12 µl of plasma and then used to deliver 5 µl each to the two diluents. This halved the loss of serum needed to wet the syringe and fill the dead space, thereby both sodium and potassium could be analysed from a total volume of 15 µl of plasma.

Serum Chloride Measurement The measurement of serum chloride was by the electrostatic (2nd) method of Ramsay et al (1955). It is an attractive method because of its simplicity of operation, because it can be used on very small samples (1×10^{-3} µl) with accuracy (better than 1%) and takes about two minutes for a complete determination.

The apparatus was built to the specifications of Ramsay et al (1955) but it was found necessary to provide extensive shieldings for cables to the meter and from the electrode system to the control box. The circuit was therefore more complex than that given by Ramsay et al (1955), see Appendix.

A direct reading transistorised volt meter was constructed to replace the Pye Universal pH meter used by Ramsay et al (see Appendix).

The concentrations of the solutions given by Ramsay et al (1955) were found to give a reference electrode system resistence of about 0.6 Mohm instead of the claimed resistence of 7 Mohms. When the solution normalities were reduced tenfold the apparatus functioned normally.

Reagents:-

1. Na NO_3 (set in 30 gms/litre agar) N/10 (N)
2. Na NO_3 solution N/10 (N)
3. Ag NO_3 solution N/10 (N)

The sulphuric acid used in the silver chloride concentration cell (the sample and acid droplet) was N/1 H_2SO_4 as stated in the paper.

Micro pipettes drawn from fine nylon cannula tubing were found to be superior to silconed vitreosil for the measurement of the calibration and serum samples. The point up to which the pipette was filled was marked with a small dot of black bitumenous paint and the samples were manipulated using pressure from the mouth. The pipettes normally held 1-2 x 10^{-3} µl.

The serum samples were titrated to an end point of 240 mV and the capacitor charges were converted to chloride concentration by comparison with the same volumes of calibration solution of known chloride content. The relationship of chloride to capacitor charge was found to be linear (Table 15) in agreement with Ramsay et al (1955). Each new pipette was therefore calibrated against a calibration solution of 100 mg/litre and a new conversion graph drawn from this point through zero.

Reproducibility using solutions of sodium chloride was very good (Table 13). Serum samples were almost as good (Table 14) but tended to block the pipette, especially after the pipette had been used for a number of samples. This problem of blockage by serum samples was not overcome, a pipette was replaced when it became blocked.

Table 13 - Series of Seven Readings on a Sample Containing 100 meq/litre Sodium Chloride

Initial Voltage mV	Condenser Charge mV
450	710
450	710
450	705
450	715
450	695
445	705
440	710

Table 14 - Chloride Values Obtained for Different Serum Samples from Rattus norvegicus

Serum Sample	Chloride Content meq/litre	Mean Chloride Content meq/litre
A	117 116	117
B	110 109	110
C	118 116	117
D	111 112 110	111
E	115	115

Table 15 - Calibration of Chloride Meter

Concentration meq/litre	Initial Voltage mV	Condenser Charge mV
100	445	500
	445	510
	450	500
	450	500
10	390	53
	395	53
	390	53

Vaginal Smears

Taking the Smear

The smears were taken with a small (2 mm diameter) loop of nichrome wire held in a dissection pin holder. The wire was cleaned and sterilised by heating to white heat for about ten seconds and then cooled by immersion in sterile physiological saline. The smear was rapidly spread on a thinly albumenised slide and fixed whilst wet.

Fixing and Staining

The smears were initially fixed and stained according to Mandl's (1951) second method. This method proved to be both prolonged and unreliable and the single differential stain and method of Shorr (1941) was subsequently adopted. Using this method the smears were fixed in a solution of equal parts of ether (diethyl ether) and 95% ethanol. The smears could remain in this fixative without affecting the subsequent procedure for a number of hours (Hartmann, 1944-45). The smears were then stained in batches, dehydrated in alcohol, cleared in xylol and mounted under cover clips in damar xylol. Ten slides could be fixed, stained and mounted in ten minutes.

Differentiation was good and there was little loss of cellular material from the slides, in consequence, there was very little interference from 'floaters' (cells crossing from one slide to another during processing).

Classification of Smears

The smears were classified as fully as possible using the classification of Mandl (1951) i.e. the transition stages were included e.g. LD/EO, EO/O etc. The identification of cell types was from the papers of Hartmann (1944-45), Mandl (1951) and Astwood (1939). The late oestrus-early dioestrus inter stage was classified as a dioestral smear (presence of leucocytes - Long & Evans, 1920).

In most cases it was found that there was a continuous presence of fully cornified epithelial cells throughout the cycle. This may well have been due to the technique used in collecting the smears and the fact that the loop method, although rapid, did not always pick up a representative sample of the lumen debris. The loop was only inserted approximately 0.5 cm into the vaginal lumen for fear of inducing pseudo-pregnancy.

The collection of debris using saline lavage was investigated but was found to be too prolonged for the present study. Although the wire loop did not provide the best collection technique and occasionally gave thin smears with very few cells it allowed the oestrous cycles of the animals in the experiments to be determined with a sufficient degree of accuracy.

C H A P T E R I V

EXPERIMENTAL RESULTS

CHAPTER IV

EXPERIMENTAL RESULTS

Evaluation and Accuracy of Analytical Methods

This section is comprised of data obtained from evaluation of the analytical methods used for determining serum osmolality and plasma sodium and potassium content. Two of the methods were sufficiently different from the normal practice to warrant trials and calibration checks.

Freezing point measurement

The accuracy of the continuous rise method (described on page 91) for determining the freezing point depends upon a slow and smooth approach to the melting/freezing point. If the rise is too fast there is the danger of over-shooting the melting point.

Smoothness of temperature rise and general response

Fig. 11. illustrates the temperature readings at ten second intervals over a period of $6\frac{1}{2}$ minutes with the mean rate of temperature increase set at 0.002C°/min - the slowest rise rate. The maximum fluctuation from the mean rate of rise was $\pm$ 0.0004°C(for the complete readout figures see appendix). The response of the bath to various conditions of heating and cooling is illustrated in Fig. 11. At a working rate of rise of 0.01C°/200 secs the delay from fast cool or fast heat was approximately ten seconds,

FIGURE 11.

Response of Bath to Controls

and Smoothness of Temperature Rise

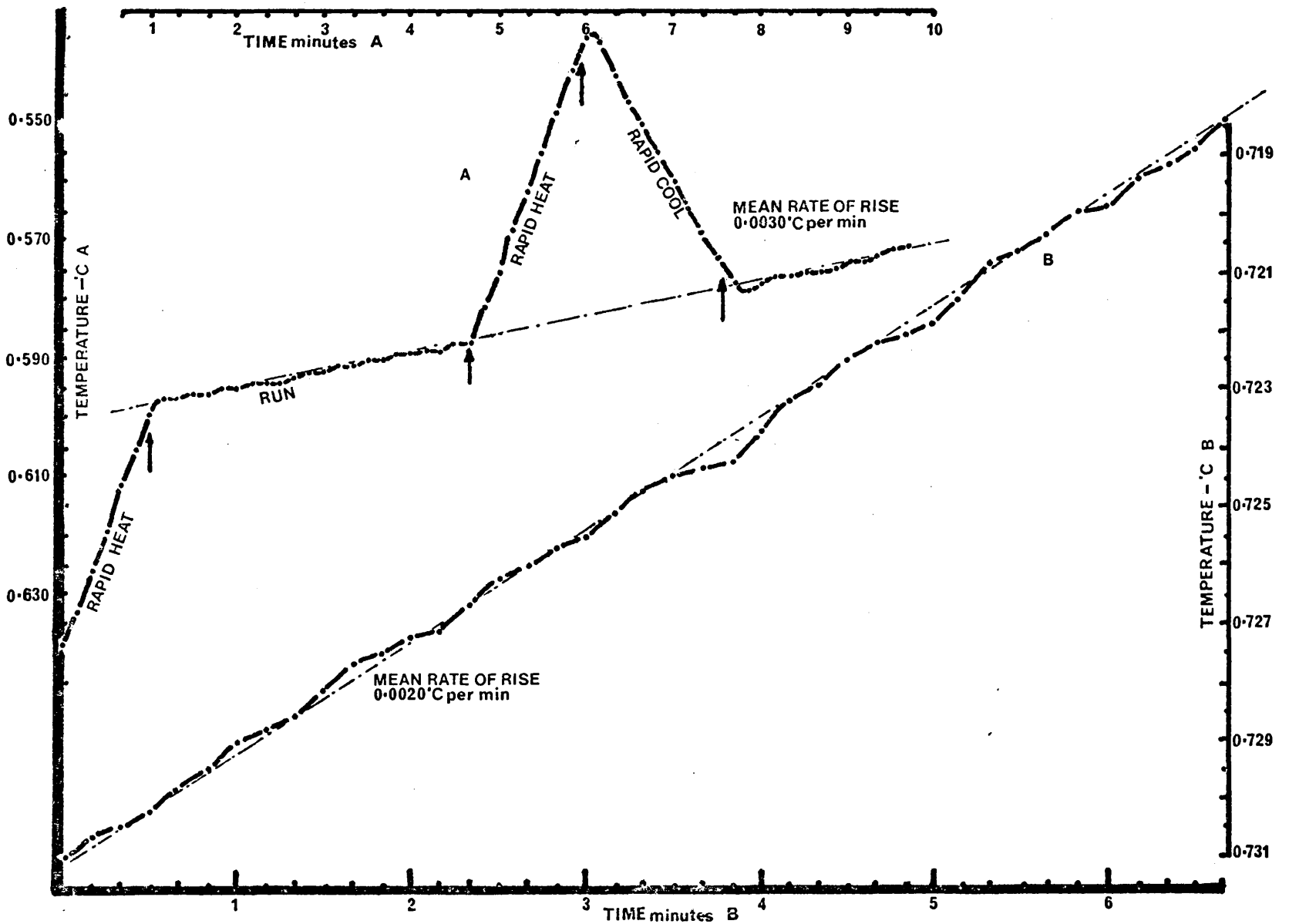


FIGURE 11. RESPONSE OF BATH TO CONTROLS AND SMOOTHNESS OF TEMP. RISE

the set rate was maintained on returning to the working rate.

The effects of rate of temperature rise and size of sample upon the observed melting point Two differently sized samples from the same solution of sodium chloride were observed end to end in the same capillary and their apparent melting points noted at different rates of temperature increase. The values obtained are shown in Table. 16. Figure 12 illustrates the values for the two samples with their regression lines (parameters of regression - Olivetti programmes T 0501).

The two regression lines differed in slope, the smaller sample A ($3.5 \times 10^{-7} mm^3$) showing a consistently greater freezing point depression. The larger sample B ($1.1 \times 10^{-6} mm^3$) showed a greater degree of variation at different heating rates and a lower value for the freezing point. This indicated that with the larger sample there was a tendency to over-shoot the melting point and this tendency increased with faster heating rates.

Reproducibility of the freezing point measurement Two series of ten trials were measured, one series with 10 samples of a solution of sodium chloride in different capillaries, Table 17, and one with 10 measurements on a single sample, Table 18.

Ten samples in ten different capillaries gave a mean value of - 0.5589°C with a standard deviation of 0.0008°C

Table (16) - Analysis of Method - Effects of sample size and rate of heating upon freezing point

	Average rate of heating near end-point x 0.0001C°/ 10 seconds	Sample A (the samples end to end in the same capillary)	Sample B
	1.5	0.5608	0.5590
		0.5608	0.5591
		0.5611	0.5597
	2.1	0.5607	0.5587
	2.5	0.5605	0.5584
	2.6	0.5604	0.5587
	3.1	0.5608	0.5587
	3.4	0.5605	0.5583
	3.8	0.5606	0.5583
	7.5	0.5600	0.5574
Mean	-	0.5606	0.5586
Standard Deviation	-	0.0003	0.0006
Standard Error	-	0.0001	0.0002

A = Sample size $3.5 \times 10^{-7} mm^3$ (1 x 1) approx

B = Sample size $1.1 \times 10^{-6} mm^3$ (1 x 4) approx

FIGURE 12.

Effects of Size & Heating

on Apparent Freezing Point

FIGURE 12. EFFECTS OF SIZE & HEATING ON APPARENT FREEZING POINT

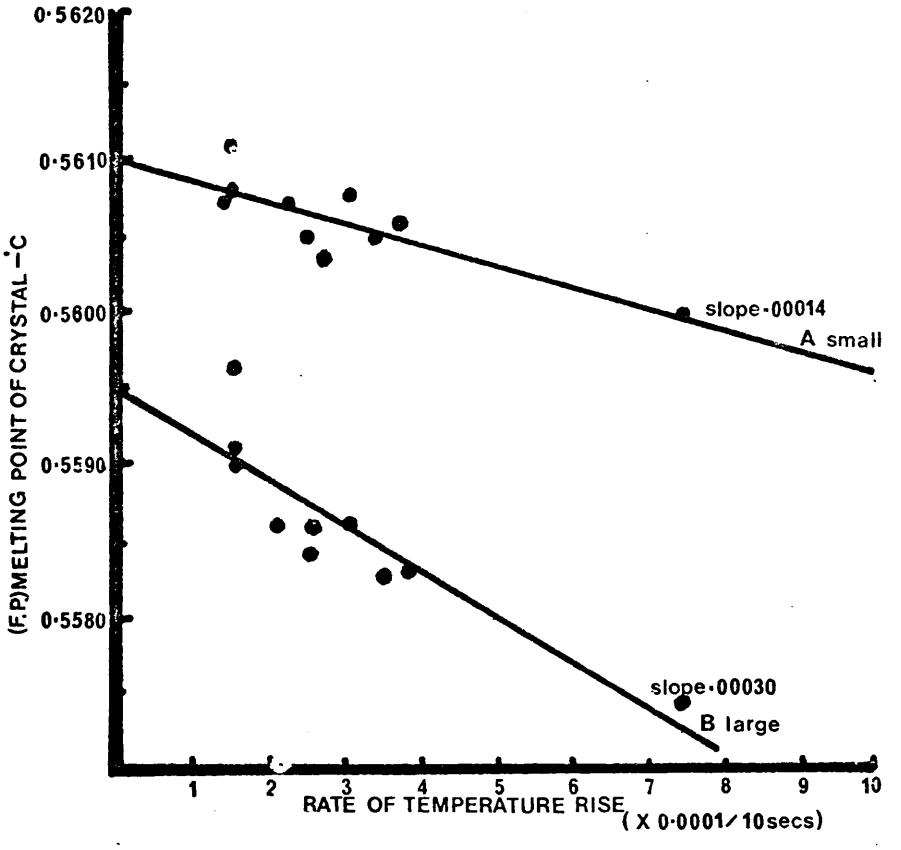


Table (17) - 10 samples of PCS in different capillaries - dimensions 1 x 2 to 1 x 4, volumes (0.8 to 1.2) x 10^{-6} mm^3

Δ -°C to 0.0001	Δ -°C to 0.001
0.5596	0.560
0.5579	0.558
0.5583	0.558
0.5600	0.560
0.5588	0.559
0.5587	0.559
0.5601	0.560
0.5582	0.558
0.5581	0.558
0.5587	0.559
Mean 0.5588	0.559
Standard Deviation 0.00079	0.00088
Standard Error 0.00026	0.00029

Table (18) - 10 consecutive readings on a single capillary sample - dimensions 1 x 4, volume c. 1 x 10^{-6} mm^3

Δ -°C to 0.0001	Δ -°C to 0.001
0.5590	0.559
0.5583	0.558
0.5584	0.558
0.5584	0.558
0.5597	0.560
0.5587	0.559
0.5587	0.559
0.5592	0.559
0.5583	0.558
0.5587	0.559
Mean 0.5587	0.559
Standard Deviation 0.00045	0.00067
Standard Error 0.00015	0.00023

and a standard error of 0.0003°C. The ten consecutive measurements on the same sample in a single capillary gave a mean of - 0.5587°C with a standard deviation of 0.0005°C and a standard error of 0.0002°C.

There was no zero drift in the two days that the experiment lasted.

It was concluded that the method was sufficiently accurate to measure the freezing point of a sample to 0.001C°.

Flame Photometry

Potassium analysis at 1:201 dilution To test the accuracy of the new method (page 109) for potassium analysis, a series of measurements were made, using reconstituted freeze-dried serum of known titre (Burroughs Welcome control serum). 5µl of the serum was diluted 1:201 with propan-2-ol diluent (for details see appendix). The results of the analysis are given in Table 19.

The mean value for recovered potassium was 5.09 mEq/litre with a standard deviation of 0.04 and a standard error of 0.02. The manufacturer's values for the potassium content of the reconstituted serum were given as 5.00 ± 0.05 mEq/litre. The mean for recovered potassium was slightly high but not significantly so (overlap of ranges). The method gave results to within 1% of the manufacturer's figures.

Table (19) - Potassium Analysis of Control Serum at 1 : 201 dilution using propan-2-ol caesium diluent

Galvanometer Deflection	Solution Potassium Content (mEq/litre)
39.0	5.05
39.0	5.05
40.0	5.15
40.0	5.15
39.0	5.05
39.5	5.10
39.5	5.10
39.5	5.10
39.5	5.10
Mean	- 5.094
Standard Deviation	- 0.0417
Standard Error	- 0.0157

Manufacturer's values for serum potassium - 5.00$\pm$0.05 mEq/l

Calibration curve for potassium Although the emission x concentration curve for aqueous solutions of potassium is linear at low concentrations of salt the extent of the linearity of the emission curve under the new conditions of an alcohol diluent was unknown.

On the assumption that interference from variable sodium content had been fully compensated for with the excess of caesium chloride in the diluent solution, the principal calibration solution (Titrisol - E Merck, Darmstadt) was diluted to the appropriate extent to provide a series of calibration solution, Table 20.

Table (20) - Calibration Values for Plasma Potassium at 1 : 201 dilution

Dilution	Equivalent potassium content mEq/litre	Readings	Mean Readings
1 : 51	14.49	104.0, 104.5, 104.5	104.5
1 : 76	9.73	75.5, 75.0, 74.5, 74.5	75.0
1 : 101	7.66	59.0, 59.0 59.0	59.0
1 : 126	6.14	48.5, 48.5, 48.0	48.5
1 : 151	5.13	39.5, 38.5, 39.0	39.0
1 : 201	3.85	set 30	30.0
Blank 0	0	set 0	set 0

Table (21) - Calibration Values for Sodium at 1 : 401 dilution

Dilution of PCS	Equivalent sodium content mEq/litre	Readings	Mean Reading
1 : 301	191.0	89.0, 88.5, 88.5	88.5
1 : 351	164.0	79.0, 79.0, 79.5	79.0
1 : 401	143.5	set 70	set 70
1 : 451	127.0	62.5, 62.5, 63.0	62.5
Blank 0	0	set 0	set 0

At low concentrations the emission curve was linear but at concentrations above 8 mEq/litre of potassium there was a pronounced curvature, most probably due to self absorption. The normal physiological range for the plasma potassium of the rat lies within the range of 5-6 mEq/litre.

Calibration curve for sodium Sodium exhibits a convex emission curve and it is necessary to use a multi-point calibration curve for the calculation of sample concentration. On the assumption that potassium did not interfere with sodium emission because of the excess caesium chloride, the calibration solutions were made by appropriate dilution of the principal calibration solution, Table 21. The normal physiological values for plasma sodium were expected to lie in the range 140-150 mEq/litre. The calibration line was slightly curved. The replicate readings were within $\frac{1}{2}$ scale division.

Changes in the Blood Composition of the Female Rat in Different Reproductive Stages

EXPERIMENT I Oestrous Cycle

There was considerable variation in the literature for the values of serum and plasma freezing point (osmolality) and peripheral haematocrit (see appendix). This experiment was conducted to ascertain the mean values and ranges, to evaluate the sampling method and to see whether daily sampling would induce anaemia.

Experimental Procedure

Nine animals were kept under the conditions of Regime I (see page 83) and sampled daily for nine days. Body weights, oestrous phase, peripheral haematocrit and serum freezing point were recorded. Haematocrit values and freezing points were determined from a single blood sample (70µl) without heparin. Vaginal smears were stained by Mandl's (1951) method.

Results

The serum freezing point The results of the freezing point determination (Table 23) were analysed by 2 way (sample day x animals) analysis of variance without replication, allowing for one missing result (days 7 An. Nos 9) (Lindquist, 1956). The results of the analysis are presented in Table 22.

Table (22)

	SS	df	MS	F	P
Animal	1389	8	174	1.023	N.S.
Day	4298	7	614	3.633	$<.01$
Animal x Day	9439	56	169		
Total	15126	71			

Conclusions:-

1. There were no significant variations between mean freezing point values for each animal.
2. There was a significant variation ($P<.01$) between mean values for each day.

Mean values for freezing points The overall mean value for the freezing point of the blood serum for 71 samples was -0.543°C with standard deviation 0.010°C and standard error 0.0011°C. The means of individual animals varied from -0.537°C to -0.553°C.

The day to day variation in freezing point The significant variation between days ($P<0.01$) indicated two things, that there were:-

1. variations due to gross technique or
2. physiological variations possibly of an environmental or of an endocrinological nature.

Table (23) - Serum Freezing Point Values (-°C) and Oestrous Cycle Phase

Animal No	DAY NO 1	2	3	4	5	6	7	8	9	Means for each animal
1	.534 OL	.522 D	-	.555 O	.530 LO/ED	.541 LD/EO	.606 O	.541 D	.539 -	.542
2	.540 (D)	.523 ED	-	.550 O	.529 LO	.541 D	.546 O	.540 OL	.543 -	.539
3	.549 DL	.514 O	-	.554 D	.537 DL	.526 D	.576 O	.548 LO/ED	.556 -	.545
4	.531 ED	.540 LD	-	.546 LO/ED	.531 LD	.536 OE	.583 O	.541 D	.544 -	.545
5	.545 LO/ED	.548 ED	-	.559 D	.579 EO/O	.548 ED	.549 D	.547 D	.542 -	.553
6	.545 OE	.549 OL	-	.547 ED	.540 D	.537 EO	.541 O	.546 D	.534 -	.542
7	.569 D	.523 D	-	.556 O	.548 O	.538 D	.536 D	.554 O	.529 -	.544
8	.538 OE	.541 LO/ED	-	.551 D	.538 O	.518 D	.546 D	.535 EO/O	.536 -	.538
9	.529 D	.537 D	-	.552 O	.543 LO/ED	.529 D	.535 O	- LO/ED	.533 -	.537
Mean values for each day	.542	.533	-	.552	.542	.535	.557	.544	.538	

Overall mean value -0.543°C SD. 0.010°C

Correlation of freezing point with oestral phase The correlation of oestrous phase (EO,O,D,LD etc.) with freezing point (Table 24) indicated that the changes were possibly cyclic in nature, the higher values seemed to occur during oestrus, whilst dioestrus seemed to be correlated with low freezing points. In Table 24 it can be seen that on days 2 and 6 where dioestrus is predominant the mean values are at their lowest values, whilst on day 7 (the only day on which there was clearly a larger number of oestrous phases) the freezing point values are at their highest.

Table (24) - Correlation of freezing point with Oestral Phase

	DAY							
	1	2	3	4	5	6	7	8
No of animals in oestrus	3	2	-	4	4	2	6	3
No of animals in dioestrus	6	7	-	5	5	7	3	5
Mean freezing point values -°C	.542	.533		.552	.542	.535	.557	.554

The freezing points were divided into two groups: 'Oestrus' (no leucocytes) or the follicular phase, and 'Dioestrus' (leucocytes present in smear), Table 25. Students 't' test for related samples was applied to the values.

Table (25) - Mean Freezing Points at Oestrus & Dioestrus

	Oestrus	Dioestrus	P
Mean freezing point values °C	-0.551±0.005	-0.539±0.002	<0.02
Osmolality (mOsmol/kg)	297±3	290±1	

Range is given as standard error of the mean

The overall mean value for freezing point and osmolality was -0.543±0.010°C and 293±5 mOsmoles/kg, which agreed fairly well with the values of -0.540°C (Maybank, 1966), 297.2 mOsmol/kg (Lichton, 1961).

The result of the test showed a significant difference ($P<0.02$) between the two sets of values.

Peripheral Haematocrit The values for haematocrit are given in Table 26. No heparin was used for the samples of blood and the results were of a limited value. The only conclusion possible was that there was no indication of anaemia i.e. the haematocrit did not fall noticeably over the period of serial sampling. There was no difference between oestrus and dioestrus. The values were rather higher than published values (see appendix) but this was to be expected because of the greater degree of plasma trapping in clotted blood. The overall mean value for haematocrit was 52% ± 2.5.

Table (26) - Peripheral Haematocrit(%)

Animal No	DAY NO 1	2	3	4	5	6	7	8	9	Means for each animal
1	49 O	56 D	- -	52 O	53 O	52 O	51 O	53 D	51 -	52
2	51 D	52 D	- -	53 O	50 O	56 D	63 O	54 O	58 -	53
3	51 D	54 O	- -	54 D	50 D	58 D	54 O	55 O	54 -	54
4	52 D	57 D	- -	57 O	53 D	55 O	52 O	53 D	48 -	54
5	57 D	53 D	- -	54 D	55 O	55 D	53 O	52 D	54 -	54
6	48 O	50 O	- -	48 D	49 D	49 O	46 O	49 D	50 -	49
7	50 D	50 D	- -	53 O	49 O	49 D	49 D	51 O	50 -	51
8	56 O	51 O	- -	53 D	55 O	52 D	51 D	52 O	52 -	53
9	50 D	51 D	- -	52 O	51 O	53 D	52 O	50 D	49 O	51
Mean of all animals each day	52	53	-	53	52	53	51	52	52	

Overall mean value 52.0% ± 2.5 (SD)

Weight Changes

It was not possible to detect cyclic changes of weight during the oestrus cycle in the present study, but this was considered to be the result of the fast.

Smears

The method of Mandl, using Schaudinn solution as a fixative, proved a very cumbersome method and not very reliable. The cells tended to float from the slide into the solutions and 'floaters' were a very big problem. It was possible however to differentiate in all cases whether the animal was in an oestrous phase or a dioestrus phase. In a few cases further differentiation was possible. The phases are shown with the appropriate serum freezing point values in Table 23.

EXPERIMENT II Premating and the First Seven Days of Pregnancy

This experiment was devised to test the improved regime (II) and the feasibility of simultaneous measurements of haematocrit, plasma sodium and potassium values. Observations were also made on the vaginal smears of premating, mating and pregnancy using the staining method of Shorr (see appendix).

Experimental Procedure

10 animals (8 mated and 2 controls) were blood sampled daily for 13 days. Oestrous phase, weight, haematocrit % and plasma sodium and potassium were recorded. Lithium heparin was used to prevent coagulation of blood samples at a concentration of 5 IU/tube. The animals were kept on grids in the boxes.

Results

Haematocrit The values are presented in Table 27. The overall mean value 47.0% (10 animals) was lower than the value obtained without heparin (52%, Results, Expt. I) but there were still fairly large day to day variations. Individuals showed similar mean values except for Nos 19 and 15.

Comparing the premating and 1st seven day values (t test related samples - Olivetti Programma program)

Table (27) - Premating, Post-mating and Control Values of Haematocrit (%) and Oestral Phase

ANIMAL	DAY OF SAMPLING 1	2	3	4	5	6	7	8	9	10	11	12	13	Mean for each animal
12	51 LD	46 EO/O	48 O	50 -	54 D	*52^{5} LD/EO	40^{5} SP	41 MD	50^{5} MD	48^{5} MD	50^{5} MD	47 MD	43 MD	49
13	55 ED/D	53 D	50 -	51 EO	*47^{5} O	50 MD	39 MD	43^{5} MD	46^{5} MD	45 MD	52 MD	48 MD	41 MD	48
14	53 O	46^{5} LO/ED	48 D	49 LD	48 LD	*42 EO/O	41 SP	45^{5} MD	49^{5} MD	46^{5} MD	50^{5} MD	45 MD	- MD	47
16	- D	46 D	46 D	50 D	44 EO	48 D	46 D	47 D	44^{5} D	45^{5} D	44^{5} D	43 D	41^{5} D	46
17	- ED	46^{5} D	47^{5} D	53^{5} D	47 D	48 LD	*49^{5} LD	50 MD	48 MD	47^{5} MD	46^{5} MD	41 MD	41 MD	47
18	52 LO/ED	49 LD	48^{5} O	53^{5} ED/D	51^{5} O	55 ED	49 D	46 LD	*43 EO	52 SP/O	45^{5} MD	45^{5} MD	- -	49
19	- LO	45 D	43 EO	47 O	44 LO/ED	36^{5} D	*44 EO	43^{5} SP	44^{5} MD	43^{5} MD	43 MD	- -	- -	44
20	50 O	49 D	48 D/LD	47^{5} LD	*49^{5} SP	49^{5} MD	52 MD	46 MD	45^{5} MD	43^{5} MD	44 MD	46 MD	- -	48
CONTROLS														
11	49 EO	- O	- LO/ED	47^{5} ED	47 EO/O	48 O	- D	48 D	48 LD/EO	41 EO/O	46 O	41 ED	41 D	46
15	- D	- D	45 EO/O	46 O	46 LO/ED	40 D	39 LD/EO	46^{5} O	41^{5} LO/ED	- D	48^{5} LD	41^{5} LD/EO	42^{5} O	44
Means for controls	49	-	45	47	46	44	39	47	45	41	47^{5}	41^{5}	42	
Daily mean all animals	52	48	47	50	48	47	46	46	47	46	47	44	41	

Overall mean haematocrit - all animals 47% ± 2.0 SD, SE 0.5

* = Time of conception

▬ ▬ ▬ ▬ = Males put with females

there was a significant difference between the two phases ($P < 0.05$), Table 28. This cannot however be attributed to the effects of pregnancy for both the controls (Nos 11 & 15) showed a similar fall in haematocrit value.

Table (28) - Haematocrit & Plasma Sodium During 1st 7 Days of Pregnancy

	Premating	1st seven days of pregnancy	P
Mean value for haematocrit, (%)	49.0 ± 0.5	46.0 ± 0.5	<*0.05
Mean value for plasma Na^+ mEq/litre	150 ± 2	149 ± 2	NS
	(35)	(36)	

Range as SE of the mean. Number in brackets is number of samples.

7 animals.

* t test related samples.

This was therefore possibly a sampling induced anaemia due probably in part to the larger number of samples taken (13) and to a regime that was too severe in its restrictions of food and water.

Plasma sodium The values are given in Table 29. The overall mean value of 149 mEq/litre agreed with published data - 152 mEq/litre (Biological Handbook), 151 mEq/litre (Spector), see appendix. The mean value for each animal fell within the expected range of 143-156 mEq/litre (Spector)

Table (29) - Plasma Sodium Values (mEq/litre)

Animal No	Day of sampling 1	2	3	4	5	6	7	8	9	10	11	12	13	Mean for each animal
12	-	-	-	-	-	154*	-	-	142	148	152	156	-	151
13	154	148	151	145	161*	-	146	147	140	146	154	137	159	149
14	176	150	156	158	158	151*	150	-	139	146	150	147	-	153
16	-	-	144	-	147	-	142	145	140	144	151	156	152	147
17	158	158	150	-	151	144	145	-	154	152	141	-	-	150
18	126	142	142	133	142	129	138	146	150*	169	123	155	139	142
19	-	145	146	-	146	151	161*	-	141	145	128	175	-	149
20	136	158	159	175*	178	170	148	143	139	152	150	151	-	155
Controls														
11	144	-	-	-	-	-	-	-	164	147	-	149	-	151
15	-	-	153	150	148	147	-	148	145	-	146	-	167	150
Daily mean all animals	149	150	150	152	154	149	147	146	145	150	144	153	154	

Overall mean value - all animals 149, SD 10, SE 1 * = date of conception

but the SD for the overall value (10) was perhaps too large. The occasional very high values and generally large fluctuations in sodium values for individuals indicated an error in the handling of the samples. There was no significant difference between the values for the premating period and the first seven days of pregnancy.

Plasma potassium The results for plasma potassium obtained for this experiment were very poor and few in number - Table 30. The method of sampling did not produce sufficient blood in many cases for both a sodium and a potassium estimation. In addition the values were high (published value is 5.9 mEq/litre (5.4 - 6.4) see appendix). It was noticed in a number of cases that there was a noticeable degree of lysis of the sample, this may have been due to insufficient heparin. Plasma storage was by deep freezing in small polythene containers without liquid paraffin and this may also have contributed to the high values.

Body weight The body weight of the control animals (Nos 15 & 11) tended to fall during the sampling period, the loss over the 13 day period was 10 gms for animal No 15 and 5 gms for animal No 11. Both animals 11 and 15 increased their weights after removal from the regime, thus indicating that the food restriction was excessive. The pregnant animals began to gain weight at approximately the same time after mating (8.7 days) as in unstressed

Table (30) - Plasma Potassium Analysis (mEqu/litre)

Animal No	Day of Sampling												
	1	2	3	4	5	6	7	8	9	10	11	12	13
11	-	-	-	-	-	-	-	10.4	-	7.3	-	-	-
12	-	-	-	-	-	-	-	-	11.7	X	X	-	-
13	6.8	7.4	7.55	7.65	-	-	8.9	9.05	9.9	9.15	9.35	8.55	9.65
14	-	8.0	10.4	8.4	10.4	8.4	-	-	9.6	8.0	10.0	-	-
15	-	-	-	-	9.9	-	-	-	11.8	11.7	-	9.3	-
16	-	-	-	-	-	9.8	11.2	-	-	-	8.6	-	-
17	-	X	-	-	-	8.45	-	-	-	7.75	-	-	-
18	-	8.5	9.8	-	-	-	11.0	-	-	-	-	10.3	-
19	-	6.9	8.0	-	10.4	-	-	-	8.1	9.4	-	-	-
20	-	10.3	11.7	-	-	-	9.9	11.6	9.0	-	8.6	X	-

animals (8.0 days). The unmated constant dioestrus animal showed a similar pattern to the controls Nos 11 and 15 with a net loss over the sampling period of 5 gms which it regained soon after removal from the regime.

Vaginal smears The oestral phases determined by the smear technique are given in Table 27. The smear of early pregnancy was typified by the presence of large numbers of leucocytes and extensively vacuolated epithelial cells of the mucous type (Type IV, Hartman) usually found on day 3 of the oestrous cycle.

EXPERIMENT III To Examine the Effects of Fasting on the Serum Freezing Point and the Plasma Sodium and Potassium Values.

The experiment was undertaken in order to find the optimum period, after commencing the fast, during which to take blood samples. The aim was to find a period of 1 hour during which any variations between animals had reached a minimum and were likely to remain steady. The parameters chosen were serum freezing point and plasma sodium and potassium.

Experimental Procedure

Four animals were kept under the conditions of regime III and sampled once daily. The heparin concentration was raised to 10IU per tube. The plasma samples were stored at -17°C to -20°C in the haematocrit tubes and analysed the following day for sodium and potassium. Vaginal smears were taken. The animals and treatments were arranged in a randomised manner.

Results

Serum freezing point The results are presented in Table 31 and graphically in figure 13. At one half hour after removal of food and water the serum freezing points showed a significant decrease; three and a half hours later the serum freezing point was essentially the

Table (31) - Serum Freezing Point, Plasma Sodium, Plasma Potassium & Oestrous Phase

Serum Freezing Point(-°C)

	hours of fast 0	½	2	4	6
41	0.553	0.535	0.545	0.546	0.557
42	0.561	0.513	0.569	0.569	0.538
43	0.558	0.539	0.511	0.547	0.573
44	0.541	0.557	0.535	0.550	0.565
Mean	0.553	0.536	0.540	0.553	0.558
SD	0.008	0.017	0.023	0.010	0.014
SE	±0.004	±0.010	±0.014	±0.006	±0.008

Plasma Sodium(mEq/l)

	hours of fast 0	½	2	4	6
41	132	135	145^5	145^5	136
42	133	139	X	142^5	147^5
43	140	137	133	134	141^5
44	143^5	145^5	139	143^5	147^5
Mean	137	139	139	141	143
SD	5.5	4.5	6.2	4.9	5.5
SE	±3.2	±2.6	±4.4	±2.3	±3.1

Plasma Potassium(mEq/l)

	hours of fast 0	½	2	4	6
41	5.95	6.40	7.10	6.35	5.30
42	X	6.85	X	6.55	6.20
43	5.50	6.35	5.15	5.65	5.80
44	5.95	6.30	6.85	6.05	5.45
Mean	5.80	6.50	6.35	6.15	5.70
SD	0.26	0.25	1.06	0.39	0.40
SE	±0.18	±0.15	±0.75	±0.22	±0.23

Oestrous Phase

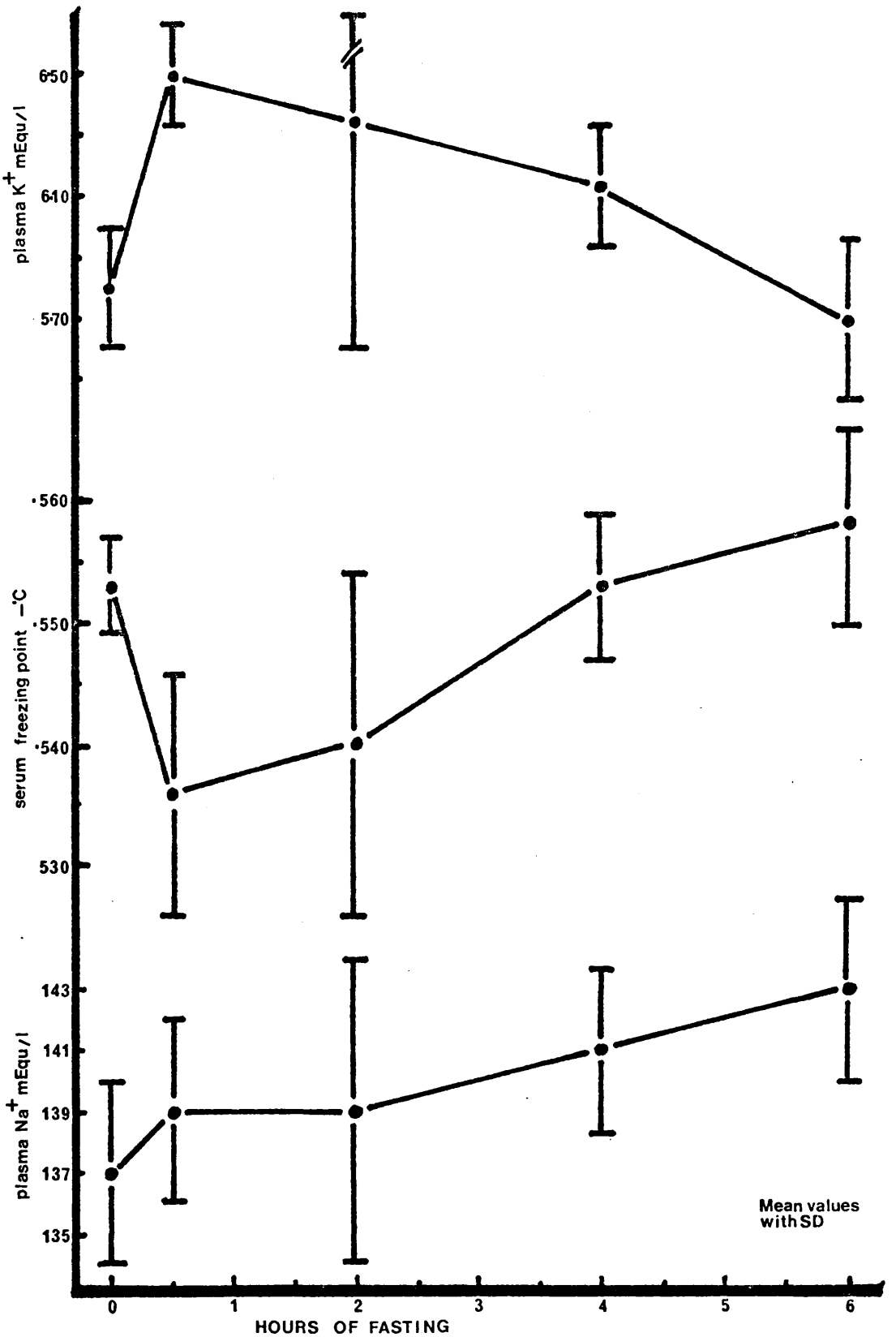
	hours of fast 0	½	2	4	6
41	D	O	D	D	LD/EO
42	D	LO/ED	D	LD/EO	O
43	EO/O	D	LO/ED	D	EO
44	LD/EO	O	O	D	LO

FIGURE 13.

Effects of Fasting on Plasma Sodium

& Potassium and Serum Freezing Point

FIGURE 13. EFFECTS OF FASTING ON PLASMA Na^+ & K^+ and SERUM F.POINT.



same as at the start of the fast. At six hours after commencing the fast there was no significant increase over the four hour level.

Plasma sodium The results are presented in Table 31 and figure 13. The plasma sodium level rose from 137 mEq/litre at the time of removal of the food and water to 143 mEq/litre six hours later. The value at six hours of fast is barely significant (SE of both values = 3) but a rise in plasma sodium could be the reason for the increased osmolality.

Plasma potassium Plasma potassium rose sharply in the first half hour (Table 31, figure 13) to a level ($6.50_{A}^{\pm}$ 0.15) mEq/l well above the average value given in the literature (5.90_{A} mEq/l, 5.6 - 6.4, Spector; 6.2_{A} mEq/l Prosser & Brown). Following this initial rise there was a tendancy for the potassium level to fall until at six hours the level was of the same order as at the start of the fast. At two hours of fast the levels were widely spread between the animals. Between four hours and six hours the potassium levels were within the normal physiological range given in the literature.

Oestral phase Comparing oestral phase and the values obtained for freezing point, potassium and sodium there was no apparent correlation between the values for these parameters and the oestral phase.

Conclusion

In all parameters there were large variations in the values for individuals at two hours after commencing the fast. In the cases of freezing point and potassium there were also sharp and significant changes at one half hour after the start of the fast.

Between four hours and six hours all parameters showed small variations between animals and for sodium and potassium approached the normal published values (potassium 5.4 - 6.4 mEq/litre; sodium 143 - 156 mEq/litre - Spector). The significance of these changes will be discussed in Chapter V.

EXPERIMENT IV The Effects of Pregnancy & Littering upon the Haematocrit, Serum Osmolality, Plasma Sodium and Potassium and Body Weight

The experiment was designed to test the improved regime III over the entire period of pregnancy and to study the feasibility of simultaneous analysis of serum freezing point, haematocrit, plasma sodium and plasma potassium. It was further hoped that more precise knowledge of the time of onset of pregnancy anaemia could be gained, with its correlation to weight gain, serum freezing point and plasma sodium and potassium.

Experimental Procedure

10 females and 5 non-mated controls selected randomly from the 15 animals, were housed in wire grid bottomed cages under Regime III. The animals were weighed daily at 13.00 hours and blood sampled every 3 or 4 days with a total of 10 samples over the entire period of premating and pregnancy and littering. 10 IU per tube of lithium heparin were used in the plasma samples.

Results

Weight gain The average weight gains for the pregnant and control animals for each day of pregnancy are given in Table 32. The average gross weight gain for 8 pregnant rats over the period of pregnancy is shown in figure 14 with the average weight gain of the controls

Table (32) - Mean Accumulative Weight Gain (in gms) of Pregnant and Control Animals During the Experimental Period

	Pregnant (8)	Control (5)
Average weight at start of exp. period	253 ± 6.5	245 ± 7.0
Premating	0 ± 3.0	
	-1 ± 5.0	-0.5 ± 1.0
	1.5 ± 2.5	- -
	4.0 ± 2.0	1.0 ± 1.5
	1.5 ± 1.5	-0.5 ± 1.5
Mating	-1.5 ± 2.0	- -
Gestation	2.0 ± 1.5	0 ± 2.0
	4.0 ± 1.5	2.5 ± 1.0
(Average time of	5.0 ± 1.5	-0.5 ± 2.0
gestation =	8.5 ± 1.5	1.5 ± 2.0
21.5 days (±0.2 SE))	8.0 ± 1.5	-0.5 ± 1.5
	9.0 ± 1.5	0 ± 3.0
	11.0 ± 1.5	-2.6 ± 1.0
	12.5 ± 2.5	-1.0 ± 2.0
	14.0 ± 2.0	-1.0 ± 2.5
	18.0 ± 2.0	-2.5 ± 2.0
	19.0 ± 1.5	-2.0 ± 2.0
	23.0 ± 2.0	-1.5 ± 2.0
	24.5 ± 2.0	-4.0 ± 2.5
	28.0 ± 2.0	-2.0 ± 2.5
	30.5 ± 2.0	-2.0 ± 2.0
	38.0 ± 2.0	-4.0 ± 2.0
	47.5 ± 3.5	-6.0 ± 1.5
	56.5 ± 3.5	-2.4 ± 3.0
	65.0 ± 5.0	-2.5 ± 1.5
	74.5 ± 5.5	-2.0 ± 3.0
	86.0 ± 6.5	-3.0 ± 2.0
Post partum	21.5 ± 3.0	-2.5 ± 2.5
	10.4 ± 4.0	-1.3 ± 3.0

Average weight of litter = 61.5 ± 5.5

Average number per litter = 11 ± 1

for comparative purposes. At day 4 the pregnant females showed the first significant weight increase over their premating levels (weight at sample 1 was taken as zero weight gain) but it was not until day 7 that there was a steady increase in weight of about 2.5 gm/day (B). At day 15 the rate of increase changed quite sharply to about 10 gm/day (C) which continued until day 21. This period of increased weight gain corresponds with the maximum growth rate of the foetuses. At the day of littering the average weight of the litters accounted for the average loss in weight of the maternal organisms. Over the same period the control animals showed no significant mean weight change.

Haematocrit The values obtained for the haematocrit are shown in Table 33, the time of mating is shown as a vertical line (M) between the figures for the mated (pregnant) group. The results for two animals (47 & 54) were separated because 47 became pseudopregnant and 54 mated very late.

The mean values for the mated and control groups were divided into five groups of means, Table 34.

These values were analysed statistically by a mixed design two way analysis of variance with replications in one factor only (Lindquist, 1956). The results of the analysis are in Table 35.

FIGURE 14.

Peripheral Haematocrit and

Weight Gain in Pregnancy

(Slopes A, B & C are the three rates of weight increase. M = time of mating, L = time of littering.)

FIGURE 14. PERIPHERAL HAEMATOCRIT AND WEIGHT GAIN IN PREGNANCY

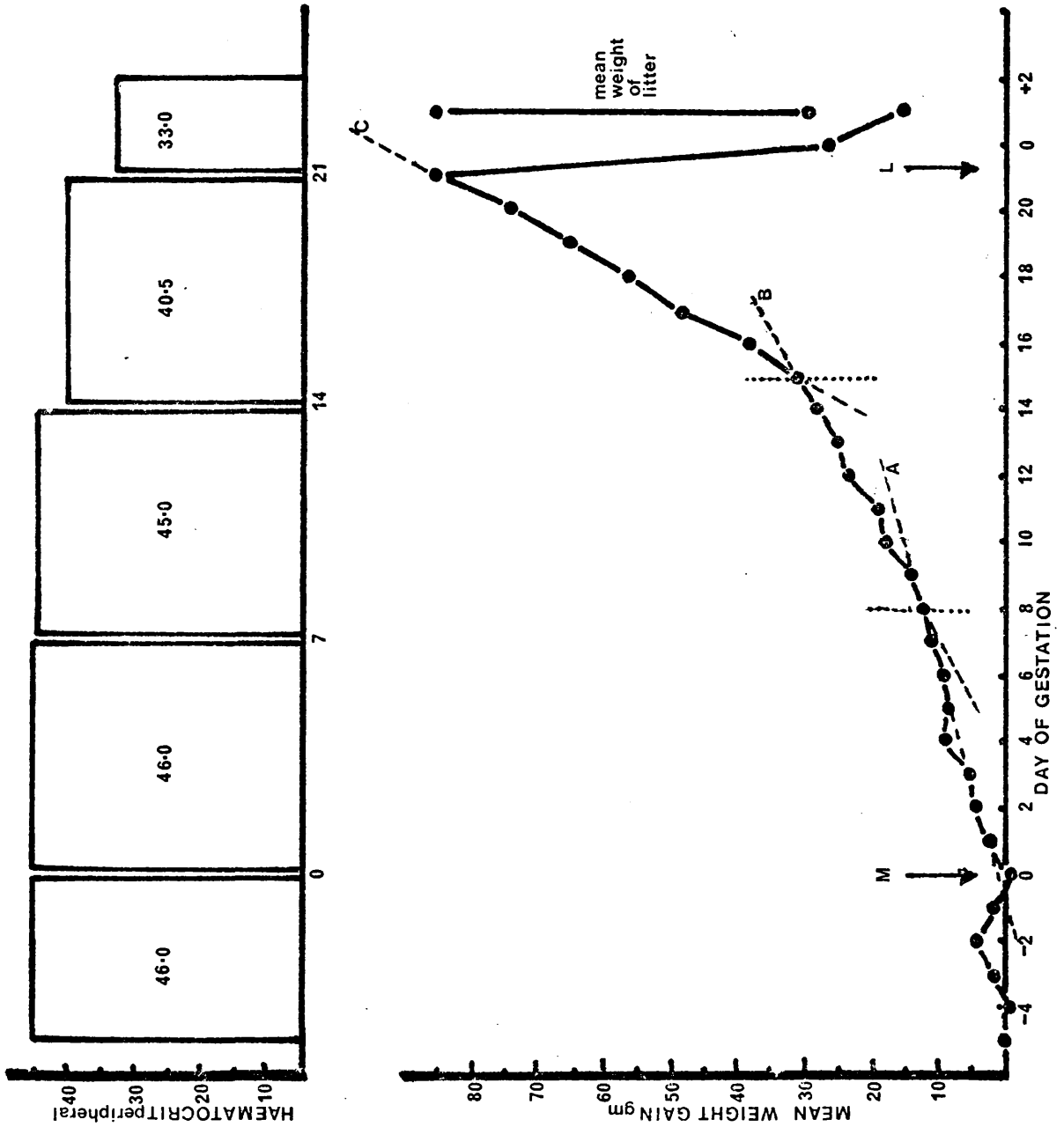


Table (33) - Haematocrit Results(%)

	SAMPLE NUMBER									
	1	2	3	4	5	6	7	8	9	10
	Pre-mating		1st 7 days			2nd 7 days		3rd 7 days		post partum
Pregnant Animals										
45	46.5	47.0	46.5	44.5	47.0	45.0	44.0	42.5	40.5	37.0
48	44.5	-	43.5	45.5	45.5	42.5	53.5	40.0	40.5	34.0
50	45.5	46.0	44.0	43.0	43.5	43.0	44.0	38.0	38.0	29.5*
52	45.5	45.0	45.5	-	48.0	43.5	49.0	43.5	44.0	34.0
53	47.5	46.5	45.0	45.0	45.5	44.5	45.0	42.0	40.0	35.0
55	47.5	45.0	45.5	45.0	43.0	42.5	45.5	43.0	40.5	37.0
57	46.0	-	44.5	48.5	49.0	-	47.5	43.5	38.0	34.0
58	48.0	46.5	45.5	50.0	47.0	50.0	48.5	42.0	33.0	22.5*
Control Animals										
46	48.0	46.5	-	47.0	48.0	46.5	45.0	47.0	46.5	40.0
49	-	-	-	-	-	-	-	-	-	-
51	42.0	42.5	43.0	45.5	46.0	47.0	45.5	44.5	45.5	45.0
56	46.5	47.0	46.0	47.0	48.0	47.5	47.0	49.0	46.0	44.0
59	46.0	43.0	48.5	48.0	47.0	43.5	48.0	47.0	45.5	45.0
Pseudo-pregnant Animals										
47	46.0	46.0	46.0	46.0	44.5	43.0	45.0	43.5	44.5	47.5
54	46.5	44.0	47.0	44.5	44.0	45.0	47.0	45.5	46.5	-

* Extensive Haemorrhage

Table (34) - Results of Haematocrit Analysis for Five Experimental Periods - data for mixed design two way analysis of variance with replications on one factor only

	Pre-mating	1st 7 days of pregnancy	2nd 7 days of pregnancy	3rd 7 days of pregnancy	post partum
Pregnant Animals					
45	47.0	46.0	44.5	41.0	37.0
48	44.5	45.0	43.0	40.5	34.0
50	46.0	43.5	43.5	38.0	29.5
52	45.5	48.0	46.0	44.0	34.0
53	46.5	45.5	44.5	41.0	35.0
55	46.5	44.5	44.0	41.5	37.0
57	45.0	49.0	47.5	41.0	34.0
58	46.5	48.5	49.0	37.5	22.5
Mean	46.0	46.0	45.0	40.5	33.0
Control Animals					
46	47.0	47.5	45.5	47.0	40.5
51	42.5	45.5	46.0	45.0	45.0
56	46.5	47.5	47.5	47.5	44.0
59	46.0	47.5	45.5	46.0	45.0
Mean	45.5	47.0	46.5	46.5	43.5

Table (35) - Analysis of Haematocrit Results

Source of variance	df	F	P
BS Between subjects	11		
B Treatments	1	26.250	<0.001
eb Error (b)	10		
WS Within subjects	44		
A Time	4	36.528	<0.001
AB Time & Treatments	4	10.349	<0.001
ew Error (w)	40		

The results of the analysis of variance were interpreted as follows:-

The A term (the variation due to time)

There was a significant variation ($P<0.001$) in the data due to time, i.e. the values for premating, the 1st seven days of pregnancy, the 2nd seven days of pregnancy the 3rd seven days of pregnancy and post partum.

The B term (the variation due to treatment i.e. control and pregnant groups)

There was significant variation ($P<0.001$) in the two sets of data, i.e. for controls and pregnant animals.

The AB Interaction

There was a significant variation ($P<0.001$) i.e. the effects of pregnancy and non pregnancy are different at different times. Testing A effect against AB effect

- divide mean square A by mean square AB = F ratio (with 4 x 4 degrees of freedom). This was not significant, i.e. the effect of A (time) was only in one of the groups, therefore during pregnancy there was a significant fall in haematocrit due to the effects of pregnancy alone.

t tests (related samples) were then performed between the different groups as follows:

a) Premating and 1st 7 days of pregnancy

b) 1st 7 days of pregnancy and 2nd 7 days of pregnancy

c) 2nd 7 days of pregnancy and 3rd 7 days of pregnancy

d) 3rd 7 days of pregnancy and post partum

The results are presented in Table 36:

Table (36) - Mean Values for Haematocrit at Five Stages

	Premating	1st 7 days	2nd 7 days	3rd 7 days	post partum
Mean haematocrit (%)	46.0	46.0	45.0	40.0	33.0
P		NS a	<0.01 b	<0.001 c	<0.001 d

The mean haematocrit, controls was 46.0 $\pm$ 0.5%

The fall in haematocrit became detectable during the second week of pregnancy. The mean fall was slight, a fall to 45% compared with 46% in the first week. During

the third week there was a pronounced fall in haematocrit to a mean value of 40.5%. Immediately post partum there was a further fall to a mean value of 33.0% due in part to the extensive parturition haemorrhage which was observed in two of the animals.

Serum osmolality (freezing point) Full figures of serum osmolality for both groups (mated and controls) are given in Table 37, with the means for each sampling day. There was a trend for the pregnant animals to exhibit lower values towards term and to rise again shortly after, the values for the controls remaining relatively constant.

A statistical analysis of the results by a mixed design two way analysis of variance with replications in one factor only (Lindquist, 1956) showed no significant variation due to treatment or time. For these results the values were grouped by animals into premating, the 1st 11 days, the 2nd 11 days of gestation and post partum. The analysis was run on all animals for premating, the 1st 11 days and the 2nd 11 days; and without number 45 on all four groups (Table 38). The means and f ratios are given in Tables 39 & 40.

With the post partum values included, the variance both among periods and treatment, closely approached the lowest level of significance and the results did not preclude a possible change in serum osmolality near

Table (37) - Results of Freezing Point Analysis(-°C)

	SAMPLE NUMBER									
	1	2	3	4	5	6	7	8	9	10
	Premating		1st half of pregnancy				2nd half of pregnancy			post partum
Pregnant Animals										
45	0.521	-	0.550	0.540	0.545	0.532	0.520	0.536	0.505	-
48	-	0.534	-	0.523	0.530	0.536	-	0.545	0.552	0.562
50	0.541	0.544	0.536	0.572	0.526	0.527	0.539	0.509	0.569	0.593
52	0.542	0.562	0.584	-	0.567	-	-	0.507	0.579	0.536
53	0.543	0.534	0.548	0.552	0.524	0.548	0.583	0.527	-	0.573
55	0.526	0.530	0.555	0.524	-	0.519	-	-	0.530	0.602
57	0.520	-	0.574	0.548	-	0.527	0.554	0.518	0.486	0.528
58	0.548	0.541	0.638	0.538	0.570	0.533	0.523	0.517	0.496	0.573
Mean	0.534	0.541	0.569	0.542	0.544	0.532	0.544	0.523	0.522	0.567
SE	0.005	0.005	0.014	0.007	0.009	0.003	0.013	0.005	0.012	0.011
Control Animals										
46	0.567	0.530	0.556	0.540	0.538	0.530	0.552	0.571	0.544	0.544
49	-	-	-	-	-	-	-	-	-	-
51	0.538	0.529	0.557	0.534	0.520	0.532	-	0.421	0.536	0.552
56	-	0.512	0.536	0.534	0.531	-	-	-	0.547	0.532
59	0.541	0.559	0.551	0.556	0.526	0.572	0.608	0.526	0.538	0.537
Mean	0.549	0.533	0.550	0.541	0.529	0.545	0.560	0.506	0.541	0.541
SE	0.010	0.011	0.005	0.005	0.004	0.017	0.040	0.037	0.002	0.004
Pseudo-pregnant										
47	0.546	0.579	0.564	0.527	-	0.537	0.546	0.549	0.548	0.544
54	0.535	0.535	0.517	0.563	-	-	0.553	0.529	0.478	-

Table (38) - Results of Freezing Point Analysis for Four Experimental Periods - data for mixed design, two way analysis of variance with replications on one factor only.

	Premating	1st 10 days of pregnancy	2nd 11 days of pregnancy	post partum
Pregnant Animals				
45	0.521	0.542	0.520	-
48	0.534	0.530	0.549	0.562
50	0.543	0.540	0.539	0.539
52	0.563	0.567	0.513	0.536
53	0.542	0.541	0.555	0.573
55	0.528	0.533	0.530	0.602
57	0.547	0.538	0.519	0.528
58	0.576	0.547	0.512	0.573
Control Animals				
46	0.551	0.536	0.556	0.544
51	0.541	0.529	0.479	0.552
56	0.524	0.533	0.547	0.532
59	0.550	0.551	0.557	0.537

Table (39) - Mean Freezing Point Values (-°C) and Osmolality (mOsmols/kg) for Four Stages

	Premating	1st 11 days	2nd 11 days	post partum
Pregnant (7)	.548	.542	.531	.567
Osmolality	295	292	285	305
Controls (4)	.542	.537	.535	.541
Osmolality	292	289	288	291

F P/C = 2.24997 i.e. P $\not<$ 0.1, NS

F periods = 2.90618 i.e. P $\not<$ 0.1, NS

Table (40) - Mean Freezing Point Values (-°C) and Osmolality (mOsmols/kg) for Three Stages

	Premating	1st 11 days	2nd 11 days
Pregnant (8)	.544	.542	.530
Osmolality	293	292	285
Controls (4)	.542	.537	.535
Osmolality	292	289	288

F P/C = 0.01737 i.e. P $\neq$ 0.1, NS

F periods = 1.4585 i.e. P $\neq$ 0.1, NS

parturition, but more values would be necessary before a definite conclusion could be drawn.

The mean freezing point for all controls (35 samples) was -0.540 ± 0.005°C as SE (291 mOsmols/kg). The standard deviation was 0.027°C, which was higher than previously (0.010°C, Experiment I).

Plasma sodium The values obtained for individual animals at different times are given in Table 41. In Table 42 the mean values for each animal in each of four time periods are given.

The mean values (Table 42) were analysed by the same two way analysis of variance (Lindquist) as before:-

1. All animals in premating, first half and second half of pregnancy
2. All animals except 53 in all four periods (no data for post partum for 53).

There was no significant variation between pregnant and control groups and no significant variation due to time, Table 43.

Table (43) - Results of Analysis of Variance

	Treatment	Time	P
1 All animals 3 periods	F = 0.18650	0.18275	NS
2 All except No 53 4 periods	F = 0.44823	1.5435	NS

Table (41) - Sodium Analysis Results (mEq/l)

	SAMPLE NUMBER									
	1	2	3	4	5	6	7	8	9	10
	Pre-mating		1st half of pregnancy				2nd half of pregnancy			post partum
Pregnant Animals										
45	140	136	143[5]	145[5]	147[5]	144	147[5]	136	140	144[5]
48	139	149	141[5]	139	137	139[5]	137	-	145[5]	145[5]
50	135	-	141[5]	132	-	145	-	137	136	150
52	141	139	143[5]	-	133	130	133	122	143[5]	145[5]
53	142[5]	134	141[5]	145	-	138[5]	-	135	133	-
55	136	135	143[5]	133	126	125[5]	126	133	147[5]	150
57	135	-	141[5]	145[5]	133	-	133	135	137	151
58	140	131	141[5]	144[5]	139	138[5]	139	139	137	127
Control Animals										
46	136	137	-	139	135	141	135	137	144	123
49	147[5]	-	-	-	-	135	-	-	-	135
51	134	141[5]	138	145[5]	135	144	135	143[5]	143[5]	149
56	143[5]	131	136	139	134	133	134	137	138	122
59	135	140	-	143[5]	138	135	138	149	147	152
Pseudo-pregnant Animals										
47	145[5]	134	141[5]	139	150	147	150	138	137	137
54	138	131	-	141[5]	137	123[5]	137	157	139	-

Table (42) - Mean Sodium Values for Four Experimental Periods of Pregnancy - data for mixed design two way analysis of variance with replications on one factor only.

Pregnant Animals	Premating	1st 10 days	2nd 11 days	post partum
45	138.0	145.0	141.0	144.5
48	144.0	139.0	141.0	145.5
50	135.0	139.5	136.5	150.5
52	141.5	131.5	133.5	145.5
53	139.5	142.5	134.0	-
55	135.5	132.0	135.5	150.0
57	138.0	139.0	135.0	151.0
58	137.5	140.5	138.5	127.0
Mean	138.5	138.5	137.0	145.0
Control Animals				
46	136.5	138.5	138.5	123.0
51	138.0	141.5	140.5	149.0
56	137.0	135.5	136.5	122.0
59	137.5	139.0	144.5	152.0
Mean	137.0	138.5	140.0	136.5

The means for the four periods are given in Table 44:

Table (44) - Mean Values for Plasma Sodium During Four Periods of Pregnancy

	Premating	1st 11 days	2nd 11 days	post partum
Pregnant mEq/litre	138.5	138.0	137.0	145.0
Control mEq/litre	137.0	138.5	140.0	138.5

On the basis of the results obtained there was no detectable change in plasma sodium due to pregnancy. The mean for the controls (41 values) was 138.5 $\pm$ 1.0 mEq/1 .

Plasma potassium All values are presented in Table 45. These were divided into four periods as in the other parameters and the means for each animal per period are shown in Table 46. The means were analysed by a two way analysis of variance with replications in one factor only (Lindquist). The analysis was run on all animals in three periods (premating, 1st 10 days and 2nd 11 days of pregnancy) and omitting no 46 (a control - insufficient values) in all four periods.

In both analyses there was no significant variation between the pregnant and control groups (F values - 0.1006 and 0.8515) although there was significant variation among the time periods in both analyses ($P < 0.01$). With treatment/

Table (45) - Potassium Analysis Results

(values in mEq/l)

	SAMPLE NUMBER									
	1	2	3	4	5	6	7	8	9	10
	Pre-mating			1st half of pregnancy			2nd half of pregnancy			post partum
Pregnant Animals										
45	5.80	6.55	6.40	5.80	7.60	7.35	7.60	6.05	6.20	7.00
48	5.55	5.80	5.70	6.55	6.25	7.35	7.70	7.20	5.40	6.00
50	6.25	-	6.55	6.55	5.80	7.60	-	6.20	5.70	5.90
52	6.85	6.00	6.45	-	6.50	5.90	-	7.70	5.25	7.30
53	6.85	6.40	6.35	6.85	7.50	6.55	-	6.55	-	8.20
55	6.85	6.25	5.60	7.20	7.35	7.20	6.55	7.75	-	8.90
57	6.10	5.95	6.65	-	7.00	7.00	7.40	5.10	-	6.35
58	6.20	5.65	6.20	7.20	6.55	8.55	6.40	-	6.30	5.65
Control Animals										
46	5.95	6.40	-	5.80	5.55	6.55	7.05	6.45	5.40	-
51	5.85	-	5.60	6.35	7.05	7.60	8.00	6.05	7.35	7.70
56	6.20	5.65	7.40	6.40	-	7.50	8.25	8.00	6.60	8.90
59	6.35	5.65	6.00	6.35	5.65	5.65	7.15	6.35	5.80	7.10
Pseudo-pregnant Animals										
47	6.20	6.45	-	7.10	6.55	7.20	6.10	6.10	7.60	-
54	7.35	6.40	-	-	-	-	6.85	6.45	-	-

Table (46) - Mean Potassium Values for Four Experimental Periods of Pregnancy - data for mixed design two way analysis of variance with replications on one factor only.

Pregnant Animals	Premating	1st 11 days	2nd 11 days	post partum
45	6.20	6.80	6.60	7.00
48	5.70	6.45	6.75	6.00
50	6.25	6.60	5.95	5.90
52	6.45	6.20	6.50	7.30
53	6.55	6.95	6.55	8.20
55	6.55	6.85	7.15	8.90
57	5.90	7.00	6.35	6.35
58	6.00	7.45	6.35	6.65
Mean	6.20	6.80	6.55	6.90
Control Animals				
46	6.20	5.95	6.30	-
51	5.70	6.80	7.15	7.70
56	6.40	6.95	7.60	8.90
59	6.00	5.90	6.45	7.10
Mean	6.00	6.40	6.90	7.90

/time interaction there was no significant variation ($F = 2.9804$) i.e. the effects of pregnancy and non pregnancy did not differ at different times.

The results of the analyses indicated that although pregnancy did not affect the plasma potassium values, some other factor caused both groups of animals to show a steady increase in values during the course of the experiment, Tables 47 & 48. The pregnant group appeared to be affected to a lesser degree.

Table (47) - Mean Values for Plasma Potassium During Three Periods of Pregnancy

	Premating	1st 10 days	2nd 11 days
Pregnant group mEq/litre	6.20	6.80	6.55
Control group mEq/litre	6.00	6.40	6.90

Table (48) - Mean Values for Plasma Potassium During Four Periods of Pregnancy

	Premating	1st 10 days	2nd 11 days	post partum
Pregnant group mEq/litre (8)	6.20	6.80	6.50	6.90
Control group mEq/litre (3)	6.05	6.55	7.05	7.90

Summary of Results for Experiment IV

1. A mean total weight gain in the pregnant animals above that of the controls became apparent at day 4 of gestation. From day 6-7 to day 15 the animals showed a steady increase in weight of about 2.5gm/day and from day 15 there was a sharp increase in weight gain to 10 gm per day which persisted to term.

2. The mean value for haematocrit fell during pregnancy from 46.0% to 40.0% with a further fall to 33.0% immediately post partum. The greatest fall occurred during the period from day 14 to parturition but a fall in haematocrit became detectable during the period day 7 to day 14.

3. There was a trend for serum osmolality to fall in the last six days of gestation but to rise immediately post partum. The changes were not significant.

4. There were no significant changes found in plasma sodium during pregnancy but there was the possibility of higher values post partum.

5. During the experiment there was a gradual and significant increase in plasma potassium which was not due to pregnancy.

The results will be discussed in Chapter V.

C H A P T E R V

DISCUSSION OF RESULTS

CHAPTER V

DISCUSSION OF RESULTS

Non Pregnant Values of Serum Osmolality

The wide differences that exist in the literature for mean plasma and serum osmolality have made it necessary to re-determine the values for the particular strain of rats used in the present study. The previous discrepancies (see appendix) were most probably due in part to the method of analysis, the figure of Collip (1920) is especially high. No specific data on the effects of fasting prior to sampling on serum osmolality were found in the literature and in view of the work of Parsons (1930) and Yin et al (1970) on the rat, and Rheinhold (1953) on the human it was expected that the environmental and nutritional conditions prevailing at sampling would effect the values obtained for plasma or serum osmolality.

A statistical evaluation of the present analytical technique showed that it was sufficiently accurate to measure the freezing point of a sample to within 0.001C° or 0.5 mOsmol/kg (Tables 16, 17 and 18). The absolute accuracy was carefully checked using the data supplied with the quartz thermometer and with calibration solutions of sodium chloride (Johnson & Hoch, 1965) - the calculations used by Johnson & Hoch are given briefly in the appendix.

Pilot studies on serum osmolality during the oestrous cycle and at intervals after commencement of a fast revealed

that both these factors significantly affected the values obtained and must therefore be taken into consideration when attempting to find the mean normal (or resting) level. The wide fluctuations in osmolality at the commencement of the fast (Table 31a) were not unexpected but subsequent to this there was a short period of lower values before the serum osmolality showed a continued and gradual increase. The rise continued until the end of the experimental period, i.e. after six hours of fasting. This gradual increase in osmolality of the serum was considered to be due to evaporative and excretory losses for it has been shown by Horowitz & Borut (1970) that evaporative and other water losses in the rat affect both the vascular and extravascular components of the extracellular space equally. In the rat the water content of the blood plasma falls as body tissue water is lost and there seems to be little or no attempt to maintain blood volume (Hainsworth 1968). The rat can exhibit considerable tolerance to plasma water loss through evaporation. The observed increase in plasma osmolality was slight, the only significant changes being between the mean values at zero and six hours and the value at half an hour (Fig 13). The values for plasma sodium tended to support the increased osmolality whilst potassium showed an inverse relationship. This may have been the result of mineralocorticoid induced retention of sodium and water in the loop of Henle with increased excretion of potassium. The changes were all

small but the experiment gave an idea of the best period in which to sample and the degree of variation to be expected at that point.

The difference between the mean values for oestrus and for dioestrus ($P < 0.02$) has not previously been noted in the rat although Paaby (1959) noted cyclical changes in plasma and serum water of the pregnant woman. These cycles corresponded closely in length with the non pregnant menstrual cycle for each subject. The mean value for oestrus (297 ± 3 mOsmol/kg) indicated a definite increase in the proportion of osmotically active particles in the plasma above the dioestrous level of 290 ± 1 mOsmol/kg The overall mean value for the non pregnant animal of 293 ± 5 mOsmol/kg (-0.543 C°) agreed with previous mean values in unpublished data (Maybank, 1966) and also agreed well with Lichton's figure of 297.2 mOsmol/kg.

There are several reasons why plasma osmolality could be higher during the oestrous phase. The high level of locomotor activity associated with the period of heat (Brobeck et al, 1947) may contribute to the increased osmolality by raising blood sugar levels (Bullough, 1949), the variation between individuals may also be due to this factor. Food intake is depressed during this period of heightened activity and this implies a change in metabolism from the dioestral phase when activity is low and food intake increases. If the high values could be more closely associated with a particular time during oestrus it would

allow a better comparison with the hormonal states, for Paaby's results certainly indicate an hormonal basis for these changes. In these results there did exist a possible correlation between early oestrus/oestrus (EO/O) and high values rather than late oestrus (LO). The changes were most probably short in duration and the smear method of determining oestrous phase could with some advantage have been replaced by sampling on a time scale.

In the period from EO to the early part of O a number of hormones reach very high but transitory levels in the blood (Fig. 5). Following a sudden increase in ovarian venous and blood plasma oestrogen there is a peak of LH and almost simultaneously a peak in ovarian venous progesterone. The peak of progesterone signals the end of water retention by the uterine tissues and the start of a period of increased metabolic activity with an increase in uterine protein and cellularity (Astwood, 1939). Energy from some source would be necessary for this increased cellular growth which coincides with the period of increased locomotor activity and lowered food intake. Progesterone has been shown to increase blood sugars (Yang, 1970) and may be the reason or part of the reason for the increase in plasma osmolality. Progesterone alone has been shown to increase the metabolic activity of uterine tissue (Saldarini & Yochim, 1967) but another hormone, growth hormone (GH) or somatotrophin, promotes general protein synthesis depending upon the direct catabolism of labile

fat stores in the liver and elsewhere (Greenbaum, 1953; Greenbaum & MacLean, 1953b). The level of GH has been shown to rise in the blood at ovulation, and premenstrually in the human, and also to increase under the influence of oestrogens and vasopressin (Spellacy et al, 1969; Frantz & Rabkin, 1965; Gagliardino et al, 1965). Although at present there is no literature available on such changes in GH in the rat it is possible that GH could be implicated in the increased metabolic activity of the uterine tissues. GH has been shown to have a secondary effect in that it is essential for the stimulation and maintenance of aldosterone production by sodium depleted rats, most probably affecting both mineralocorticoid and glucocorticoid production (Palmore et al, 1970). Thus an increase in plasma GH associated with a changing uterine metabolism could be one reason for increased plasma osmolality due to sodium retention induced by increased aldosterone production.

During the early part of EO there is an increase of tissue water in the uterus under the influence of oestrogen which rises to high levels in the ovarian venous plasma only during this period (Yoshinaga et al, 1969). The cells of the uterus accumulate water and presumably electrolytes. It is possible that the fluid later moves out of the cells and into the lumen to form the fluid suggested by Long & Evans to be of value in supporting spermatozoan activity, i.e. there is a movement of water into the cells and then out of the cells. Glucocorticoids such as

methyl-prednisolone have been shown to increase the extracellular fluid compartments of the rat. Simultaneously with the change in compartment volumes due to the methyl-prednisolone, the osmolality of the plasma has been seen to rise although the plasma sodium remained normal (Moses, 1965). The mineralocorticoid, deoxycorticosterone, also increased the plasma volume and raised the osmolality. Aldosterone being a mineralocorticoid with glucocorticoid properties may well have a similar effect. It was suggested by Moses that glucocorticoids might increase the osmotic coeficient of intra-cellular particles. The changes found in plasma osmolality by Moses (303 - 306 mOsmoles/kg) were of the same order as those found in this study (290 - 297 mOsmoles/kg).

The periods of E0 and 0 in the female rat are periods of rapidly changing hormonal and metabolic states with considerable movement of water into and out of tissues. It is suggested that the raised serum osmolalities found during this period were a reflection of increased corticosteroid secretion in which GH was implicated. Until more closely spaced data is available to show exactly when these changes occur during the oestrous cycle it is impossible to differentiate between the effects due to simple dehydration, those of increased locomotor activity and those due to the fundamental hormonal control of the oestrous cycle.

Changes in Haematocrit and Serum Osmolality During Pregnancy

Peripheral haematocrit fell perceptibly during the second week of pregnancy from a non-pregnant mean value of 46.0% to a mean value of 45.0% ($P < 0.01$). During the first seven days there was no significant change from the non-pregnant mean value of 46.0% and the change noticed during the second week was slight and did not occur in all the animals., Table 33. Throughout the third week however all animals exhibited a pronounced drop in haematocrit but again the changes differed between animals. The mean value for this period was 40.5% ($P < 0.001$). Post partum there was a further fall in haematocrit, the two animals that showed very low values bled noticeably at parturition - the mean for the short period post partum was 33.0% ($P < 0.001$). These results agree basically with the data collected by Beaton *et al* and Van Donk *et al* although their values for the non-pregnant animals are considerably higher (50%). Both used an anticoagulant but measured the haematocrit of venous blood either from the heart or by decapitation, this would account for the difference between their results and those of this study, where the haematocrit was measured on samples of peripheral blood. Serial sampling of peripheral blood for haematocrit on a microscale was therefore considered to be as accurate as previous methods.

The general pattern of anaemia followed the changing pattern of foetal and placental growth and blood volume

(Csapo & Wiest, 1969; Beaton et al, 1954; Bond, 1948; Newcomer, 1947). The start of the large fall in haematocrit in the last week of pregnancy and the start of the great increase in rate of foetal growth occurred simultaneously, that is, after day 14 and before day 17. Comparing the values for haematocrit with those of the total mean weight gain by mother plus foetuses, there was a close correlation between anaemia and rate of increase in weight (Figure 14). During the second week the mean weight gained per day was raised slightly above that of the first week to about 2.5 gm/day, but from day 14-15 there was a large increase in the daily weight gain (about 10 gm/day), ie. the weight gain appeared to be bi-phasic or possibly tri-phasic. The above values for the weight gain in the first two weeks of pregnancy are in agreement with the pattern of moisture and fat storage found by Beaton et al where there was a slight increase during the first week and a greater increase during the following week.

In the human the anaemia of pregnancy has been found to be partially due to a disproportionate increase in blood plasma volume. There is so far no indisputable proof that this is the case in the rat. That the two species may be totally different in this respect is shown by data that suggests an increase in plasma osmolality immediately prior to term in the human (Paaby, 1959) and a decrease in the rat (Lichton, 1961).

Bond noted a post partum drop in blood volume without a drop in the erythrocyte or haemoglobin levels immediately post partum. This is a clear indication that his measurement of blood volume measured the plasma volume of mother plus foetuses. Until maternal blood volume is measured using a dye that does not readily cross the placenta, such as T1824 (Evans Blue), there is no direct evidence for an increase in the maternal blood volume. Fruhman (1968) using T1824 has reported an increase in plasma volume in the mouse during pregnancy, as the mouse and the rat are very alike in their reproductive physiology it might indicate that this would occur in the rat. Newcomer (1947) noticed an increase in the vascularity of the uterus during the later stages of pregnancy and it is usually the case that, in order to maintain venous return, the blood volume expands to fill the system (Gregersen & Rawson, 1959).

The serum osmolality showed a trend towards lower values near term with a rise immediately post-partum, Tables 3, 37, 38, 39 & 40. These results although not statistically significant are basically in agreement with the findings of Lichton (1961) who found a reduction in plasma osmolality at term. Further support is provided by the data of Van Donk et al (1934) for refractive index and sedimentation rate, Figure 3. The failure of the present results to show a significant fall was most probably due

to experimental error or the large degree of individual variation, this observation is based on the f ratios which are just below the lowest level of significance, Table 39.

The blood volume has not been shown to increase with theoccurence of the anaemia but by term the mean total extracellular space of dam and foetuses (as SCN space) has increased from 31.2% v/w of the body weight to 37.3% That this is the case during the later part of pregnancy also is indirectly indicated by the observations of Bond and the reports of general hydration (Van Donk et al, Beaton et al). Part of the mechanism for this may be the drop in blood proteins leading to an increase in extra-vascular fluids and possibly an oedemic condition: Van Donk et al noted an increase in the water content of skin and muscle at this time. Bond's hypothesis that there was an increase in red cell production during the later stages of pregnancy was based upon his measurement of blood volume, which was inaccurate. On the basis of reticulocyte counts there is no evidence to suggest a significant increase in erythropoesis during pregnancy (Van Donk et al). In recent work on the mouse a large increase in young reticulocytes was found between days 9 and 15 and in both the rat and the mouse the spleen shows an increase in weight during the first half of pregnancy and then a decrease (Fruhman, 1968; Abramson, 1934). In themouse this pattern of weight change

can be correlated to the erythropoitic activity of the spleen and follows the blood reticulocyte levels. The difference between the animals may be a matter only of degree, the smaller animal with its higher metabolic rate may respond more noticeably.

The influence of the placenta upon the anaemia and the anaemia producing effects of oestrogen have been shown by Newcomer. On day 14 of pregnancy the concentration of oestrogen in ovarian venous blood has been shown to rise from non-detectable levels and was maintained at 0.5 ng/ml plasma and although this level was low compared with the levels of the oestrous cycle (c. 4.0 ng/ml plasma) there was a considerable increase in ovarian blood flow at this stage of pregnancy, c. 4.0 ml/ov/30 min, compared with 2.5 ml/ov/30 min (Yoshinaga et al, 1969). There was a further increase from day 20 to parturition. This period of increased oestrogen production by the ovaries coincided both with the onset of increased placental growth with its own production of oestrogen and with the decrease in splenic size. It is quite possible that during early pregnancy there is increased erythropoiesis which later slows down. Thus if an increase in blood volume were to occur in early pregnancy as suggested by Bond it would be counter-balanced by increased blood cell formation. Later, due to placental and ovarian interference, erythropoiesis may slow down whilst plasma volume still increases. On the

other hand the red cell numbers may remain static and the plasma alone may increase in volume. The alternative to the above hypothesis is a decrease in erythropoiesis. Further work in the manner of Fruhman is needed to help elucidate this problem.

The lower protein content of the plasma earlier proposed as a factor responsible for the increase in extravascular fluid could also be responsible for the steady rise in GFR during gestation (Lichton, 1963). Such an increase in GFR at a time when the foetuses need water and electrolytes would necessitate increased tubular reabsorption. If the loss of carcass moisture and lowered plasma sodium at term are an indication, even the reabsorption mechanisms are barely sufficient in the last day or so. It has been suggested that the two pre-requisites for parturition are an increase in uterine volume and low blood progesterone levels (Gorski, 1968). Unfortunately the results for sodium analysis do not support Lichton's work for sodium at term but this may be due to analytical technique or the change may be quite brief and vary among animals. The increased sodium retention during the last week of pregnancy reported by Lichton may be due in part to the corticoid stimulating effects of GH. Apart from the retention of sodium and the retention and deposition of protein at the expense of maternal fat stores, the liver urea formation (Q urea) and alanine-glutamic transaminase activity fall during the last week of gestation; a similar

reaction to that evoked by injection of GH into the normal animal (Beaton et al).

The values obtained for plasma sodium and potassium for the pregnant animals showed no significant changes from the non-pregnant controls throughout pregnancy and it is therefore unlikely that they had a substantial effect upon the serum osmolality. The trend towards a lower osmolality was most probably due to the lower amino acid content of the blood.

In both controls and pregnant animals there was a gradual increase in the potassium level throughout the experimental period (Tables 45, 46, 47 & 48). The increase was proportional in both cases and was most probably due to sampling. The problems of storing small samples will be discussed later.

Anaemia followed very closely the total maternal weight increases and pattern of foetal and placental growth. The anaemia began during the second week but only became apparent in all animals between the day 14 and 17, the time of a great increase in foetal and placental growth and at the commencement of protein retention by the maternal body. The anaemia was considered to be caused, at least in part, by a disproportionate increase in plasma volume and was possibly aggravated in the later stages by a drop in erythropoiesis due to increased oestrogen production by the placenta and by the ovaries. Changes in the composition

of the maternal body in the last week of gestation indicated a rise in GH secretion which was probably also responsible for the increased retention of sodium and water during this period. In as much as serum osmolality is an indication of water content some support was given to the hypothesis of an hydraemia in the later stages of pregnancy. There was no indication that either sodium or potassium played any role in the lowered osmolality which was possibly due to a lower amino acid content. Further work on plasma electrolytes and water content is needed.

The Use of Serial Sampling and Micro Analysis

The question finally arises of the validity of serial sampling and the use of micro-analytical techniques. The accuracy of the micro techniques has been shown for osmolality (to within 0.001°C), haematocrit (to within 0.5%), plasma sodium (to within 0.5 mEq/litre), plasma potassium (to within 0.05 mEq/litre) and plasma chloride. The methods were simple to use but did need practice to achieve reproducible results. Their main advantage is that they enabled serial sampling of blood to be used without adversely affecting the animal, always providing that the sampling and environmental conditions were correct. The low mean values for plasma sodium (138.5 $\pm$ 1.0 mEq/litre compared with 140-145 mEq/litre, see Appendix) do however,

illustrate the difficulties of attempting to compromise between the conditions necessary for a stable relationship between plasma and erythrocytes for two electrolytes that behave in diametrically opposed ways e.g. it was impossible to prevent the sodium transferring to the cells whilst preventing the leakage of potassium out. In future work blood samples for sodium analysis must be separated from those for potassium and spun down in cooled centrifuges at 0 °C, the small size of the sample in this study allowed the blood to rapidly warm up in the centrifuge. The speed of separation, approximately 10-15 minutes, was not short enough to prevent some exchange of sodium. The problem arose because the separation of red cells was directed towards keeping the much lower plasma potassium levels static.

The rise in plasma potassium in both controls and pregnant animals throughout the experimental period was an unexpected result. The rise was significant ($P < 0.01$) but no explanation can be given for this phenomenon.

Two remaining problems are those of sampling and storage. For haematocrit and osmolality the tail-vein sampling method gave excellent results but, until the storage problem can be overcome it is difficult to decide what effect this method of collection had on plasma sodium and potassium. The use of lithium heparin at a level of 10 IU per tube seemed necessary to prevent lysis and gave

good results initially in experiment IV, continued sampling at the same spot may have been the reason for the increased potassium levels found later.

Prolonged storage (that is for a week or more) at -20°C in the hard glass tubes was observed to increase both the potassium and the sodium values in a variable manner. The samples were analysed within 24 hours but some contamination from the glass may have occurred. Although the glass tubes were subjected to prolonged soaking after the degreasing treatment it may have been necessary to give them a dilute hydrochloric acid wash prior to the soak to ensure complete removal of adsorbed metallic ions. Substitution of nylon or vitreosil tubes for the hard glass ones may have been another solution but this was not investigated.

Micro techniques for analysis are suited to chronic studies in small mammals and involve few problems in themselves. The biggest problem is the correct handling of the sample between sampling and analysis; this has been mostly overcome but it still remains necessary to improve the method of storage, should storage be necessary.

REFERENCES

REFERENCES

ABELE, J. E. (1933) The physical background to freezing point osmometry and its medical-biological applications. Am. J. Med. Electron. 2: 32-41.

ABRAMSON, M. (1934) The effects of pregnancy on the organ weights of the albino rat. Am. J. Obstet. Gynec. 27(4): 492.

AMENOMORI, Y., C.L. CHEN and J. MEITES (1970) Serum prolactin levels in rats during different reproductive states. Endocrinology 86: 506.

ARMSTRONG, D. T., K. A. KNUDSEN and L. S. MILLAR (1970) Effects of prolactin upon cholesterol metabolism and progesterone biosynthesis in corpora lutea of rats hypophysectomised during pseudopregnancy. Endocrinology 86: 634-641.

ASTWOOD, E. B. (1939) Changes in the weight and water content of the uterus of the normal adult rat. Am. J. Physiol. 126: 162-170.

BALL, J. (1937-38) A test for measuring sexual excitability in the female rat. Comp. Psychol. Monogr. 14: 1-37.

BARRACLOUGH, C. A. and E. W. HALLER (1970) Positive and negative feedback effects of oestrogen on pituitary LH synthesis and release in normal and androgen sterilised female rats. Endocrinology 86: 542-551.

BEATON, G. H., J. BEARE, M. H. RYU and E. W. McHENRY (1954) Protein metabolism in the pregnant rat. J. Nutr. 52: 291-304.

BEILIN, L. J., G. J. KNIGHT, A. D. MUNRO-FAURE and J. ANDERSON (1966) The measurement of sodium concentration in human red blood cells. J. gen. Physiol. 50: 61-74.

BIOLOGY DATA BOOK, Ed. Altman & Dittmer, Federation of American Societies for Experimental Biology, Washington D. C. (1964).

BLAND, K. and B. T. DONOVAN (1965) in: Advances in Reproductive Physiology Vol I page 194. Logos-Academic.

BOND, C. F. (1948) The nature of the anaemia of pregnancy in the rat. Endocrinology 43: 180-186.

BROBECK, J. R. (1945) Effects of variations in activity, food intake and environmental temperature on weight gain in the albino rat. Am. J. Physiol. 143(1): 1-5.

BROBECK, J. R., M. WHEATLAND and J. L. STOMINGER (1947) Variations in regulation of energy exchange associated with estrus, diestrus and pseudopregnancy in rats. Endocrinology 40(2): 65-72.

BULLOUGH, W. S. (1949) The relation between the epidermal mitotic activity and the blood sugar level in the adult male mouse, Mus musculus L. J. exp. Biol. 26(1): 83-99.

BURHOE, S. O. (1940) Heart sampling, methods of securing blood from rats. J. Hered. 31:445-448.

BURRIEL-MARTI F. and J. RAMIREZ-MUNOZ (1957) Flame Photometry - a manual of methods and applications Elsevier press.

CANTARROW, A. and M. TRUMPER (1963) Clinical Biochemistry, 3rd edn. Wiley-Interscience, New York,

COLDMAN, M. F. and W. GOOD (1967) The distribution of sodium, potassium and glucose in the blood of some mammals. Comp. Biochem. Physiol. 21(1): 201-206.

COLLIP, J. B. (1920) Osmotic pressure of serum and erythrocytes in various vertebrate types as determined by the cryoscopic method. J. biol. Chem. 42: 207-212.

COOPER, K. and N. HAYNES (1969) Modifications of the oestrous cycle of the underfed rat associated with the time of feeding. J. Reprod. & Fert. 19:577-579.

CRAMPTON, E. W. and L. E. LLOYD (1954) The effect of water restriction on the food intake and food efficiency of growing rats. J. Nutr. 52:221-224

CRESKOFF, A. J. (1942) Haematology of the rat in laboratory investigations. Ed. Friggith and Farris.

CSAPO, A. I. and W. G. WIEST (1969) An examination of the quantitative relationship between progesterone and the maintenance of pregnancy. Endocrinology 85: 735-746.

CUSHNIE, G. H. (1954) from The care and management of laboratory animals. U.F.A.W. Handbook, 2nd Ed.

D'AMOUR, F. E., F. R. BLOOD and D. A. BELDEN (1965) Manual for laboratory work in mammalian physiology 3rd Ed. Chicago U.P., Chicago.

DEAN, J. A. (1960) Flame photometry. McGraw-Hill, New York.

DEMPSEY, E. W. and H. R. SEARLES (1943) Environmental modification of certain endocrine phenomena. Endocrinology 32(2): 119-128.

DORCHE, M. J. and C. COSTET (1955) Sur l'eau distillée et les récipients utilisés en spectrophotométrie de flamme. Bulletin de la société de pharmacie de Bordeaux, 94: 18-20.

EVERETT, J. W. (1945) The microscopically demonstrable lipids of cyclic corpora lutea in the rat. Am. J. Anat. 77:293-323.

EVERETT, J. W. (1948) Progesterone and estrogen in the experimental control of ovulation time and other features of the estrous cycle in the rat. Endocrinology 43: 389-405.

EVERETT, J. W. and T. TEJASEN (1967) Time factor in ovulation blockade in rats under differing lighting conditions. Endocrinology 80(4): 790-792.

EMERY, F. E. and E. L. SCHWABE (1936) The vaginal smears of rats as influenced by frequent examinations. Anat. Rec. 64(2) Suppl. 2: 147-154.

FAWKE, L and P. S. BROWN (1970) Pituitary content of follicle-stimulating hormone in the female rat. J. Reprod & Fert. 21:303-312.

FINK, G., R. NALLAR and W. C. WORTHINGTON (1967) The demonstration of LH releasing factor in hypophysial-portal blood of pro-estrous and hypophysectomised rats. J. Physiol. 191: 407-416.

FOLLEY, S. J. and A. L. GREENBAUM (1947) Changes in the arginase and alkaline phosphatase contents of the mammary gland and liver of the rat during pregnancy, lactation and mammary involution. Biochem. J. 41: 261-269.

FRANTZ, A. G. and M. T. RABKIN (1965) Estrogen stimulation of growth hormone release. J. clin. Invest. 44: 1049.

FRUHMAN, G. J. (1968) Blood formation in the pregnant mouse. Blood 31(2): 242-248.

FUNDER, J. and J. O. WIETH (1966a) Determination of sodium, potassium and water in human red blood cells. Scand. J. clin. Lab. Invest. 18:151-166.

FUNDER, J. and J. O. WIETH, (1966b) Potassium, sodium and water in normal human red blood cells. Scand. J. clin. Lab. Invest. 18: 167-180.

FUNDER, J. and J. O. WIETH (1967) Trapping of sodium, potassium, sucrose and albumin in the packed cell column of the haematocrit. Acta physiol. scand. 71:105-112.

GAGLIARDINO, J. J., J. D. BAILEY and J. M. MARTIN (1967) Effects of vasopressin on serum levels of HGH. Lancet 1: 1357-8.

GALETTI, F., and A. KLOPPER (1962) Influence of the administration of progesterone on the quantitiy and distribution of body fat in the female rat. Excerpta Medica, International Congress series, International congress on hormonal steroids 51.

GILLISEN, G. and H. B. GROENEVELD (1966) from Cryobiology. Academic Press. Ed. H. T. Merryman.

GOODMAN, J. R., J. VINCENT and I ROSEN (1954) Serum potassium changes in blood clots. Am. J. clin. Pathol 24(1): 111-113.

GORSKI, R. A. (1968) The neural control of ovulation. in Biology of Gestation Vol I, Ed. N. S. Assali. Academic Press, New York & London.

GREGERSEN, M. I. and R. A. RAWSON (1959) Blood volume. Physiol. Rev. 39:307-342.

GREENBAUM, A. L. (1953) Changes in body composition and respiratory quotient of adult female rats treated with purified growth hormone. Biochem. J. 54: 400-406.

GREENBAUM, A. L. and P. McLEAN (1953a) The mobilisation of lipid by anterior pituitary growth hormone. Biochem. J. 54: 407-413.

GREENBAUM, A. L. and P. McLEAN (1953b) The influence of pituitary GH on the catabolism of fat. Biochem. J. 54:413-423.

GREENWALD, G. S. (1966) Ovarian follicular development and pituitary FSH and LH content in the pregnant rat. Endocrinology 79: 572-578.

HAINSWORTH, F. R. (1967) Saliva spreading, activity and body temperature regulation inthe rat. Am. J. Physiol. 212(6): 1288-1292.

HAINSWORTH, F. R. (1968) Evapourative water loss from rats in the heat. Am. J. Physiol. 214(5): 979-982.

HAINSWORTH, F. R., E. M. STRICKER and A. N. EPSTEIN (1968) Water metabolism of rats in the heat: dehydration and drinking. Am. J. Physiol. 214(5): 983-989.

HAM, A. W. (1965) Histology 5th Ed. Pitman Medical Publ. Co., London.

HAMILTON, T. H., C. C. WIDNELL and J. R. TATA (1967) Metabolism of ribonucleic acid during the oestrous cycle. Nature, Lond. March 11: 992-994.

HARTMAN, C. (1944) Some new observations on the vaginal smear of the rat. Yale J. Biol. Med. 17: 99-112.

HASHIMOTO, I., D. M. HENDRICKS, L. L. ANDERSON and R. M. MELAMPY (1968) Progesterone and pregn-4-en-20α-ol-3-one in ovarian venous blood during various reproductive states in the rat. Endocrinology 82(2): 333-341.

HEALD. P. J., J. E. O'GRADY and A. J. HOWIE (1970) Quantitative changes in uterine nucleic acids during the oestrous cycle in rats. J. Reprod & Fert. 21:481-488.

HENDRY, E. B. (1962) The osmotic pressure and chemical composition of human body fluids. Clin. Chem. 8(3): 246-265.

HERLYN, U., H. F. GELLER, L. VON BERSWORDT WALLRABE and R. VON BERSWORDT WALLRABE (1965) Pituitary lactogenic hormone release during onset of pseudopregnancy in intact rats. Acta endocr., Copenh. 48(2): 220-224.

HERRMANN R., (1953) Flammen photometrische ultramicroanalyse von Na, K und Ca im serum. Zeitschrift für die gesamte experimentelle medezin, 122: 84-89.

HOLSINGER, U. W. and J. W. EVERETT (1970) Ovarian thresholds to exogenous LH at varying times in the rat oestrous cycle. Endocrinology 86(2): 257-260.

HORI, T., M. IDE and T. MIYAKE (1968) Ovarian estrogen secretion during the oestrous cycle and under the influence of exogenous gonadotrophins in rats. Endocr. Jap. 15: 215-222.

HOROWITZ, M and A. BORUT (1970) Effect of acute dehydration on body fluid compartments in three rodent species Rattus norvegicus, Aconys cahirinus and Meriones crassus. Comp. Biochem. Physiol. 35: 283-290.

HOUTSMULLER, A. J. (1959) Uptake of potassium by erythrocytes in relation to their glucose consumption. Clinica chim. acta. 4: 606.

HULTMAN, E. and J. BERGSTRÖM (1962) Plasma potassium determination. Scand. J. clin. Lab. Invest. 14 Suppl. 64: 87-93.

HYTTEN, F. E. and G. A. CHEYNE (1962) The distribution of thiocyanate in the product of conception. Clin. Sci. 23: 125-128.

HYTTEN, F. W. and I. LEITCH (1964) The physiology of human pregnancy. Blackwell Scientific Publications, Oxford.

JOHNSON, R. B. and H. HOCH (1965) Osmolality of serum and urine from 'Standard methods of clinical chemistry' Vol 5. Ed. Meites, S. Academic Press.

KASSAL, S. O. and S. LEVITAN (1953) A jugular technique for the repeated bleeding of small animals. Science, N.Y. 118: 563-564.

KINGSLEY, G. and R. SHAFFERT (1954) Micro flame photometric determination of sodium, potassium and calcium in serum with organic solvents. J. biol. Chem. 206: 807-815.

LICHTON, I. J. (1961) Salt saving in the pregnant rat. Am. J. Physiol. 201: 765-768.

LICHTON, I. J. (1963) Urinary excretion of water, sodium and total solutes by the pregnant rat. Am. J. Physiol 203(4): 563-567.

LINDQUIST, E. F. (1953) Design and analysis of experiments in Psychology and Education. Houghton Mifflin Company, Boston, Massachusettes.

LONG, J. A. and H. EVANS (1922) The oestrous cycle in the rat and its associated phenomena. Mem. Univ. Calif. 6: 1-148.

MACINTYRE, I. (1961) Flame photometry. Advances in Clinical Chemistry 4: 1-27.

MAHLER, H and E. CORDES (1968) Biological Chemistry. Pub. Harper & Row, New York & London.

MAIZELS, M and M. REMINGTON (1959) Percentage of intercellular medium in human erythrocytes centrifuged from albumin and other media. J. Physiol., Lond. 145: 658-666.

MANDL, A. (1951) The phases of the oestrous cycle in the adult white rat. J. exp. Biol. 28: 576-584.

MATHER, A. and N. R. MACKIE (1960) Effects of haemolysis on serum electrolyte values. Clin. Chem. 6(3): 223-227.

MAYBANK, G. T. (1966) Honours thesis. Unpublished, University of Aberdeen, Scotland.

MAYBANK, G. T. (1970) A simple flame photometric method for the estimation by emission of potassium in 5µl of whole blood plasma, using the Zeiss PMQ II with FA2 Burner and the M4Q III single monochromator. Zeiss Mitt. Fortschr. tech. Opt. 5(5): 243-247.

MONROE, S. E., R. W. REBAR, V. L. GAY and A. R. MIDGLEY (1960) Radioimmunoassay determination of luteinising hormone during the oestrous cycle of the rat. Endocrinology 85: 720-724.

MOSES, A. M. (1965) Influence of adrenal cortex on body water distribution in rats. Am. J. Physiol. 208(4): 662-665.

NEWCOMER, J. S. (1947) The relation of the hyphysis, ovary, placenta and foetus to the development of anaemia during the last half of pregnancy in the rat. Endocrinology 40: 182-191.

NJAA, L. R., F. UTME and O. R. BREAKKAN (1957) Effect of relative humidity on rat breeding and ringtail. Nature, Lond. 180: 290-291.

PAABY, P. (1959) Changes in the water content of serum and plasma during pregnancy. Acta. obstet. gynec. scand. 38: 297-314.

PAABY, P. (1961) Changes in concentration of water in plasma during pregnancy. Munksgaard, Copenhagen.

PALMORE W. P., R. ANDERSON and P. J. MULROW (1970) Role of the pituitary in controlling aldosterone production in sodium-depleted rats. Endocrinology 86: 728-734.

PARSONS, H. T. (1930) Urea concentrations in the blood of the rat in relation to pregnancy and lactation on diets containing varying concentrations of protein. J. biol. Chem. 88:311-330.

PAULY, J. E. and L. E. SCHEVING (1965) Daily leucocyte rythms in normal and hypophysectomised rats exposed to different environmental light-dark schedules. Anat. Rec. 153: 349-360.

PIACSEK, B. E. and J. MEITES (1965) Reinitiation of gonadotrophin release in starved rats by constant light or adrenaline. Abstract from Programme of the 47th meeting of the Edocrine Society, p 117.

PIKE, R. L., H. B. SUDER and M. L. ROSS (1954) The influence of protein and energy intakes upon nitrogen retention in the pregnant rat. J. Nutr. 52: 297-309.

PONTEN, U. and B. K. SIESJÖ (1967) Acid-Base relations in arterial blood and cerebrospinal fluid of the unanesthetised rat. Acta physiol. scand. 71: 89-95.

PROSSER, C. L. and F. A. BROWN (1962) Comparative animal physiology. Saunders, Philadelphia & New York.

PRUGGER, H. The development of absorption flame photometry to a chemical laboratory technique. Zeiss information leaflet No 56, S50-654-e.

RAJ, H. G. M. and N. R. MOUDGAL (1970) Hormonal control of gestation in the intact rat. Endocrinology 86(4): 874-889.

RAMIREZ, V. D. and S. M. McCANN (1964) Fluctuations in plasma luteinizing hormone concentrations during the estrous cycle of the rat. Endocrinology 74(5): 814-816.

RAMIREZ-MUNOZ, J. (1968) Atomic absorption spectroscopy and analysis by atomic absorption flame photometry. Pub. Elsevier Press, New York.

RAMSAY, J. A. (1949) A new method of freezing point determination for small quantities. J. exp. Biol. 26(1): 57-64.

RAMSAY, J. A. (1950) The determination of sodium in small volumes of fluid by flame photometry. J. exp. Biol. 27: 407-419.

RAMSAY, J. A. and R. H. J. BROWN (1955) Simplified apparatus and procedure for freezing point determinations upon small volumes of fluid. J. scient. Instrum. 32(10): 372-375.

RAMSAY, J. A., R. H. J. BROWN and P.C. CROGHAN (1955) Electrometric titration of chloride in small volumes. J. exp. Biol. 32(4): 822-829.

RAMSAY, J. A. R. H. J. BROWN and FALLOON (1953) Simultaneous determination of sodium and potassium in small volumes of fluid by flame photometry. J. exp. Biol. 30(1): 1-17.

REINHOLD, J. G. (1953) in Standard methods of clinical chemistry. Ed. M. Reiner. Academic Press, New York. 1: 65-70.

ROTHCHILD, I (1965) Interrelations between progesterone and the ovary, pituitary and the central nervous system in the control of ovulation and the regulation of progesterone secretion. Vitams. Horm. N.Y. 23: 210.

RUGH, R. and C. SOMOGYI (1969) The effect of pregnancy on peripheral blood in the mouse. Biological Bulletin 136: 454-460.

SALDARINI, R. J. and J. M. YOCHIM (1967) Metabolism of the uterus of the rat during early pseudopregnancy and its regulation by estrogen and progestogen. Endocrinology 80: 453-466.

SAMSON WRIGHT (1966) Samson Wright's Applied Physiology, revised by Keele, C and Neil, E, 11th Ed. Oxford Medical publications.

SCHALCH, D. S. and S. REICHLIN (1966) Plasma GH in the rat determined by radioimmunoassay: influence of sex, pregnancy, lactation, anaesthesia, hypophysectomy and extrasellar pituitary transplants. Endocrinology 79: 275-280.

SCHLENKER, F. S. and J. NOLL (1952) On the determination of packed cell volume. J. Lab. clin. Med. 39(4): 582-594.

SCHREIBER, M and C. A. ELVEHJEM (1954) The influence of flavonoid compounds on the nasal excretion of a red pigment by rats subjected to stress conditions. J. Nutr. 52: 257-270.

SIEBERT, H. and S. RAPPOPORT (1955a) Über die emissionssteigernde Wirking von Electrolyten und Methanol bei der flammen photometrischen Kaliummanalyse. Biochem. Z. 326: 413-419.

SIEBERT, H and S. RAPPOPORT (1955b) Zur flammenphotometrischen Kalium bestimmung im Blutserum: verwendung emissionssteigernder Zusätze. Das Deutsche Gesundheitswesen. 13: 481-484.

SHAW, J. (1954) A simple procedure for the study of ionic regulation in small animals. J. exp. Biol. 32(2): 321-329.

SHORR, E. (1940) A new technique for staining vaginal smears:II. Science 91: 321.

SHORR, E. (1941) A new technique for vaginal smears:III, a single differential stain. Science N.Y. 94: 545-546.

SMIT, J., C. ALKEMADE and J VERSHURE (1951) A contribution to the development of the flame-photometric determination of sodium and potassium in blood serum. Biochim. biophys. Acta 6: 508-523.

SPECTOR (1956) Handbook of Biological Data. W.B. Saunders (publ.) Philedelphia & London.

SPELLACY, W. N., W. C. BUHI and R. P. BENDEZ (1969) Human growth hormone levels during the menstrual cycle. Am. J. Obstet. Gynec. 104: 1138-1142.

SQUIBB, R. L., M. A. GUZMAN and N. S. SCRIMSHAW (1954) Effect of high environmental temperatures on metabolism, I Growth and blood constituents of rats exposed to 94°F for 72 hours. J. Nutr. 52(2): 155-163.

SURE, B., M. C. KIK and D. J. WALKER (1929) Blood formation of the albino rat on a standard dietary regime. (Research paper No 91, Journal Series, University of Arkansas) J. Nutr. 1:299-310.

TOTTON, M. (1959) Ringtail in newborn Norway rats, a study of the effects of environmental temperature and humidity on incidence. J. Hyg., Camb. 56: 190-192.

VAN DONK, E. C., H. FELDMAN and H. STEENBOCK (1934) An analysis of the anaemia of pregnancy in the rat. Am. J. Physiol. 107(3): 616-627.

VARLEY, H. (1963) Practical Clinical Biochemistry. 3rd Edition. Heineman.

VAZQUEZ, O. N., K. NEWERLY, R. S. YALOW and S. A. BERSON (1952) Determination of trapped plasma in the centrifuged erythrocyte volume of normal human blood with radioiodinated (I^{131}) human serum albumin and radioserum (Na^{24}). J. Lab. clin. Med. 39(4) 595-604.

VON BERSWORDT-WALLRABE, R. and C. W. TURNER (1961) Influence of lactogenic hormone and estradiol benzoate upon placentoma formation in intact rats measured by total DNA. Proc. Soc. exp. Biol. Med. 108: 212-214.

WALKER, J and W. H. GERARDE (1966) An ultramicro method for determining sodium and potassium in serum or plasma. Am. J. med. Technol. 32(4): 251-254.

WALSH, A. (1955) The application of atomic absorption spectra to chemical analysis. Spectrochim. Acta 7: 108-117.

WESTHUES, M. and R.FRITSCH (1964-65) Animal Anaesthesia, Vol. II, translated by A. D. Weaver. Oliver & Boyd.

WINSTEN, S. (1965) Collection and preservation of specimens; in Standard methods of clinical chemistry, Ed. S. Meites, Vol. 5. Academic Press.

WOLDRING, M. G. (1953) Flame photometric determination of sodium and potassium. Analitica chim. Acta 8: 150.

YAMAMOTO, W. S. and J. R. BROBECK (1965) Physiological controls and regulations. Bicentennial volume, Univ. of Pennsylvania School of Medicine, Department of physiology. Saunders, Philadelphia.

YANG, M. M. (M. YANG MEI-PO) (1970) Effect of a single dose of progesterone on blood glucose in rats. Endocrinology 86(4): 924-927.

YIN, T. H., C. L. HAMILTON and J. R. BROBECK (1970) Effect of body fluid disturbance on feeding in the rat. Am. J. Physiol. 218(4): 1054-1059.

YOSHINAGA, K., R. A. HAWKINS and J. F. STOCKER (1969) Estrogen secretion by the rat ovary in vivo during the estrous cycle and pregnancy. Endocrinology 85: 103-112.

ZEILMAKER, G. H. (1963) Experimental studies on the regulation of corpus luteum function in castrated male rats bearing a transplanted ovary. Acta endocr. Copenh. 43(2): 246-254.

ZEILMAKER, G. H. (1965) Normal and delayed pseudopregnancy in the rat. Acta endocr. Copenh. 49L 558-566.

ZEISS (1968) Flame photometric serum analysis. Carl Zeiss, Oberkochem, Germany. GA 50-657/8/Flaz/med/11e.

A P P E N D I X

Appendix A - Values for Sources of Error in Temperature Measurement

Error factor	Error of reading		
	Stated value	Value in this application	Remarks
Linearity	0.002°C/ 10°C span	negligible	linearity error decreases with reduced span
Hysteresis	0.001°C/ 10°C range	negligible	hysteresis error decreases with reduced range
Response time to step change in probe temp.	99.9% of final value reached in 14.0 secs	possibly negligible (see remarks)	not stated for a continuous rise in probe temperature
Stability			
a. Short term	$<$ 0.0001°C/ reading	$<$ 0.0001°C/ reading	S.D. of indicated value
b. Zero-drift	$<$ 0.00001°C/ hour	$<$ 0.00001°C/ hour	usually negative in sign; calibrate to zero periodically
c. Ambient temperature changes	$<$ 0.002°C/ °C change in ambient; $<$ 0.001°C if oscillator in ice bath.	of the region of $\pm$ 0.001°C	environment of oscillator kept steady

Conditions of application:-

Measurement of continuous temperature rise of 0.003°C/min, working span of 0.2C° at -0.560. 10 sec. readings, sample temperature is approached from same direction each time.

From Hewlett Packard (Technical Data)

Appendix B

Calculation of Osmolality From Freezing Points

Although in some fields of research the expression of colligative properties in the units of freezing point depression (Δ_f°C) is the accepted way to present results it is sometimes useful to represent the results simultaneously in the form of the osmolality of the solution. This may be defined as the molality of an ideal substance dissolved in water in amounts sufficient to produce the same freezing point depression as the specimen produces (Johnson & Hoch, 1965). There is considerable confusion in terminology, the osmolality inferring the molal glucose equivalent (Wolf, 1966) whereas in practice osmolality is determined by comparing its freezing point with that of an NaCl solution of known osmotic pressure.

Wolf suggests the use of the term osmosity which is exactly a molar NaCl equivalent i.e. the osmosity of a solution is then exactly equal to the molarity of an NaCl solution, having the same freezing point, the point being that it is easier to manipulate volumetric (molar) solutions than molal solutions. It is the convention in biological systems however, to use the term osmolality which is expressed in milliosmoles per kilogram of water, thus 1 mOsmol kg^{-1} gives a freezing point depression of 0.001858°C.

Due to ionic and molecular interaction in an aqueous solution of NaCl the relationship between molality and freezing point is non linear and follows a gentle curve. In practice there is therefore a discrepancy between the theoretical (Δt) and actual freezing points (Δa). The ratio $\Delta a/\Delta t$ is the osmotic coeficient ϕ and varies considerably at low molality for sodium chloride solutions. In the preparation of solutions of sodium chloride for calibration purposes the equation

$$C = 0.1086 \frac{O}{K}$$

should be used (Johnson & Hoch), where C is the number of grams of NaCl to be dissolved in 1 kg of water, O is the required value for the milliosmolality, and K is the freezing point lowering per unit molality for sodium chloride at the particular milliosmolality. K is given in degrees C/moles per kilogram H_2O. The constant 0.1086 is one thousandth of the product of the molecular weight of sodium chloride and the molal freezing point depression of water, 1.858°C.

The values for the constant K (or 1.858 nϕ where n = number of dissociation particles) used by Fiske (cited Johnson & Hoch) were superceded by new values which were used by Johnson & Hoch to recalculate the amounts of sodium chloride necessary to give the required milliosmolality.

On the basis of 1 mOsmol kg^{-1} giving a freezing point depression of 0.001858°C in an aqueous solution, the

milliosmolality of a solution is therefore given by the equation

$$\text{Milliosmolality} = \frac{\text{observed freezing point depression } (^{\circ}C)}{0.001858}$$

which is compatible with other authors. However, for thermometric calibration purposes the modified Johnson & Hoch values in the following table have been used, which will give values for milliosmolality slightly higher (in this range c. - 300 mOsmols/kg than those where the Fiske values have been used.

Table

Standard (Millios-moles/kg H_2O)	Expected Freezing Point Lowering (Δ,°C)	Recalculated for nominal value of Fiske (g NaCl/kg H_2O)
100	0.186	3.094
300	0.557	9.476
400	0.745	12.700

Appendix C

Solutions for Potassium Analysis at 1 : 201

Diluent

1. To 1 gm of reagent grade caesium chloride in 1 litre class A volumetric flask was added the contents of a diluent ampoule (Titrisol No 9979).

2. The diluent was made up to the mark with a solution of 60% v.v. reagent grade propan-2-ol in deionised water. The diluent was stored in a screw top polythene bottle.

Calibrant

5 µl of Principal calibration solution was added from a 25 µl Hamilton syringe to 1 ml of the above caesium/alcohol diluent.

Sample

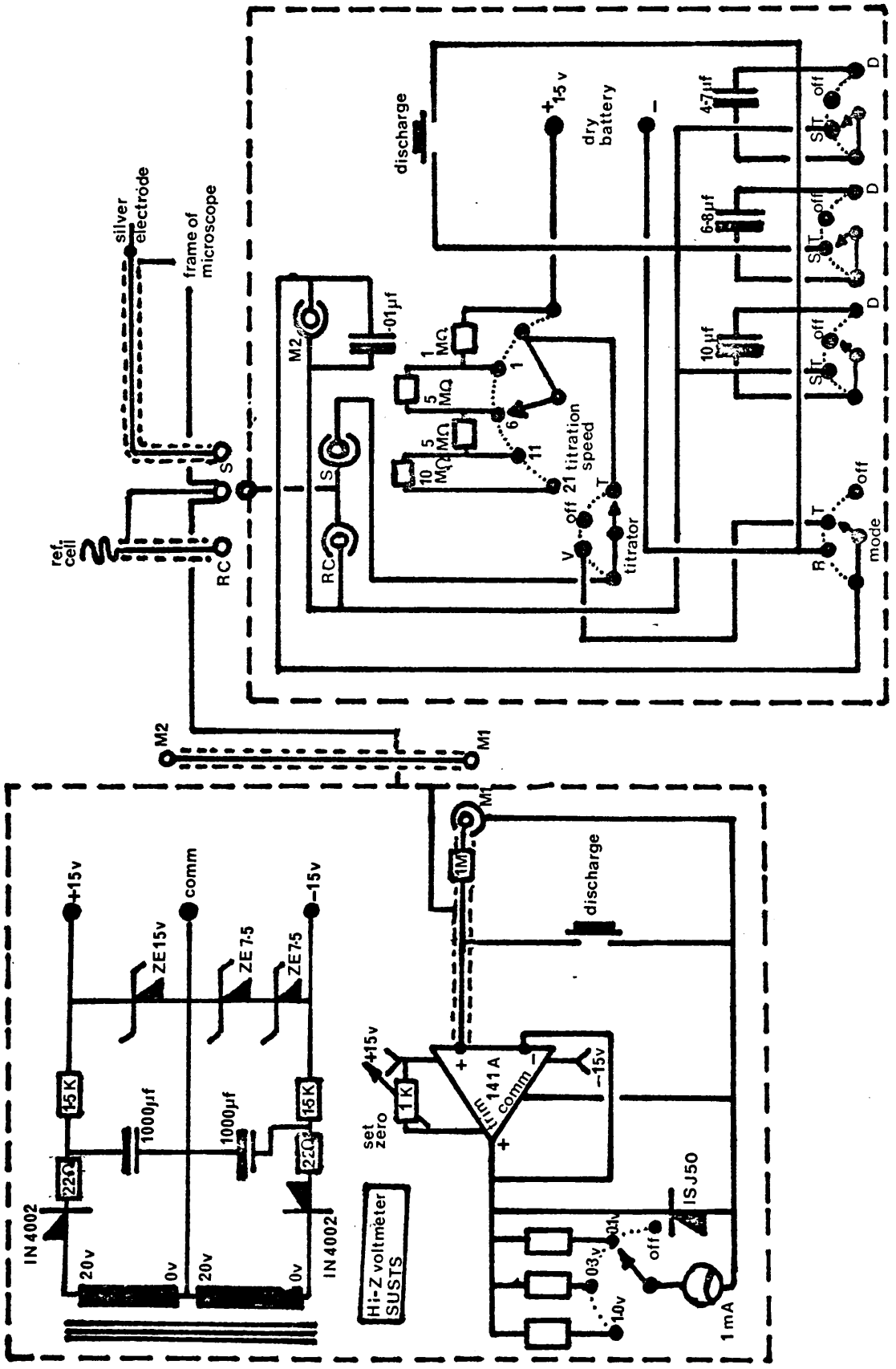
5 µl of plasma was added from a 25 µl Hamilton syringe to 1 ml of caesium/alcohol diluent.

Blank Caesium/alcohol diluent

APPENDIX D

Circuits for Chloride Meter

APPENDIX D CIRCUITS FOR CHLORIDE METER



Appendix E

A Single Differential Stain - Shorr S_3

ethyl alcohol (50%)	100 cc
Biebrich Scarlet (water soln.)	0.5 gms
Orange G	0.25 gms
Fast green FCF	0.075 gms
phosphotungstic acid c.p.	0.5 gms
phosphomolybdic acid c.p.	0.5 gms
glacial acetic acid	1.00 cc

(The solution should not be used until all the ingredients have dissolved completely).

a. Stain for approximately 1 minute in S3

b. Carry through 70%, 95% and absolute alcohol, dipping slide 10 times in each solution.

c. Clear in xylol and mount in damar-xylol.

Fixative:- equal parts 95% ethanol and diethyl ether - 1 minute minimum.

Coating of slides with albumen

1% w.v. solution of dried egg albumen in distilled water and 1 mg of phenol/100 ml as preservative. Degrease and clean slides, dip in above solution, drain and air dry vertically at room temperatures away from dust.

Appendix F

Some Values for the Blood of Rattus norvegicus

Parameter	Sample	Value	Author
Blood volume (ml/100 gm body weight)	-	7.6 - 7.8	Bond (1948)
	-	5.6 - 7.0	Biology Data Book (1964)
	-	5.7	Prosser & Brown (1962)
	-	6.0	D'Amour & Blood (1965)
Haematocrit (P.C.V.%)	-	43.4±3.1	Coldman & Good (1967)
	peripheral	46 (39-53)	Biology Data Book (1964)
	venous	50.3 (42.3 - 61.5)	" " " "
	-	39.6±0.6	Moses (1965)
	venous	48.2	Beaton et al (1954)
Osmolality (mOsmol/kgH_2O)	serum	260 (220 - 290)	Spector (1956)
	plasma	303.6-0.8	Moses 1965)
	serum	303 - 310	Yin et al (1970)
	serum	297.2±0.84	Lichton (1961)
Freezing point (-°C)	serum	0.540	Maybank (1966)
	serum	0.619	Collip (1920)
Glucose (mg/100 ml)	whole blood	56 - 76	Spector (1956)
	whole blood	69 ± 7	Coldman & Good (1967)
	plasma	107 ± 17	" " "
	cells	21 ± 9	" " "
Sodium (mEq/litre)	plasma	141.6±10.8	Coldman & Good (1967)
	plasma	144 (134 - 155)	Biology Data Book (1964)
	serum	151 (143 - 156)	Spector(1956)
	-	145	Prosser & Brown (1962)
	plasma	145.0±1.9	Moses (1965)
	serum	140.5±0.68	Lichton (1961)
	serum	140.6-139.8	Yin et al (1970)

Potassium/...

Parameter	Sample	Value	Author
Potassium (mEq/litre)	plasma	4.2±0.5	Coldman & Good (1967)
	plasma	5.9	Biology Data Book (1964)
	serum	5.9	Spector (1956)
	-	6.2	Prosser & Brown (1962)
	serum	4.6	Yin et al (1970)
Chloride (mEq/litre)	serum	110	Spector (1956)
	plasma	118	Biology Data Book (1964)
	-	116	Prosser & Brown (1962)
	plasma	103.6	Ponten & Siesjö (1967)

REPRINTED FROM ZEISS-MITTEILUNGEN **5** (1970), No. 5

G. T. MAYBANK

A Simple Flame Photometric Method for the Estimation by Emission of Potassium in 5 µl of Whole Blood Plasma, Using the ZEISS PMQ II with FA 2 Burner and the M4Q III Single Monochromator

Edited by CARL ZEISS Oberkochen / Germany

DK 535.33 : 612.12

(From the Department of Biology, University of Stirling, Stirling, Scotland)

A Simple Flame Photometric Method for the Estimation by Emission of Potassium in 5 µl of Whole Blood Plasma, Using the Zeiss PMQ II with FA2 Burner and the M4Q III Single Monochromator

By G. T. MAYBANK

A method is described for the estimation by flame emission of potassium in 5 micro litres (µl) of blood plasma at a dilution of 1 : 201. The method uses an iso-propanol/cesium/water diluent and the use of commercially available ampoules makes the method both simple and accurate. A concise method and settings are given.

Eine einfache flammenphotometrische Methode zur Wertbestimmung von Kalium in Emission an 5 µl gesundem Blutplasma mit dem Zeiss PMQ II mit FA 2 Brenner und M4Q III Einfach-Monochromator.

Eine Methode zur Wertbestimmung von Kalium in 5 µl Blutplasma in Flammenemission bei einem Verdünnungsverhältnis von 1 : 201 wird beschrieben. Das Lösungsmittel besteht aus Isopropanol, Caesium und Wasser. Die Verwendung handelsüblicher Ampullen macht das Verfahren einfach und genau. Die Methode wird genau beschrieben und Einstellungen werden angegeben.

Une méthode photométrique simple à flamme pour la détermination de la valeur de potassium sur 5 µl de plasme sanguin avec PMQ II Zeiss avec brûleur FA 2 et monochromateur simple M4Q III.

Une méthode pour la détermination de la valeur de potassium dans 5 µl de plasme sanguin en émission de flamme avec un rapport de dilution de 1 : 201 est décrite. Le solvant consiste en isopropanol, césium et eau. L'utilisation d'ampoules commerciales rend le procédé simple et précis. La méthode est décrite en détail et des réglages sont indiqués.

The monochromator used was the standard model with quartz prism and a wave length range of 185 mµ to 2.5 µ. The detector unit was used in conjunction with a pen recorder. The air compressor supplied with the PMQ II was fitted with a more efficient water trap.

Blood plasma potassium analysis at a dilution of 1:201 had advantages in that very small samples could be used and the physical characteristics of

plasma and standard were more closely matched. Excessive dilution, however, could have produced results that would have been incorrect (HOLIDAY & PREEDY) and the degree of ionisation of potassium ions would also have been increased (DEAN). Furthermore, the low emission obtained would have resulted in small detector readings and therefore a high reading error. The standard analytical method recommended (ZEISS) for serum analysis was that of HERMANN for which ampoules were available commercially [1]. This method was recommended for a maximum dilution of 1 : 50. It was quite a simple matter to extend this method to enable it to be used for a dilution 1 : 201.

The blood was collected directly into 50 µl glass microhaematocrit tubes [2], treated with 10 I. U. per tube of lithium heparin [3]. The blood was centrifuged at 12,000 G for five minutes and the first 1 mm of plasma was discarded with the red cells. Treatment of the blood with a suitable anticoagulant was found to be necessary to prevent clotting and lysis in the small bore tubes. Lithium heparin allowed the plasma to be used for the determination of the other important cations: sodium, calcium and magnesium. 5 µl of the heparinised plasma was diluted 1 : 201 with an alcohol diluent for analysis of potassium.

Spectral interference was prevented by using the strongly emitting red resonance doublet at 766 mµ and 769 mµ. This doublet, when used in conjunction with a narrow spectral slit width of 30 mµ or less, removed sodium interference at 818 mµ to 819 mµ (DEAN). Flame background was very low at this part of the spectrum (DEAN) and the narrow slit width reduced it

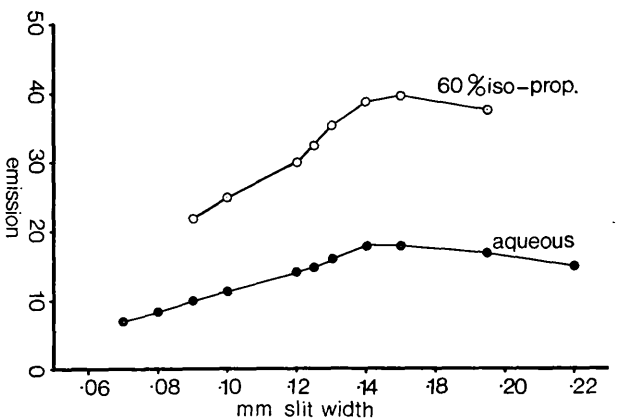


Figure 1. Emission at optimum gas settings and max. possible amplification, blank as zero. (3.85 meq/l. 1 : 201 K^+)

further. A slit width of .14 mm to .15 mm was found to give optimum results (Fig. 1.), i. e. a spectral band of approximately 15 mμ. The greater ionisation of the potassium atoms at low concentration markedly reduced the number of available neutral atoms that could be excited and thus reduced the emission (DEAN). This produced a concave emission curve, which was overcome by the addition of cesium chloride [4] and the use of a cool flame i. e. air-hydrogen; the result was a strengthened or flat emission curve (Fig. 2).

The use of a very narrow slit width reduced the emission to the unacceptably low value of 18 scale divisions (Fig. 1) and to increase the emission an

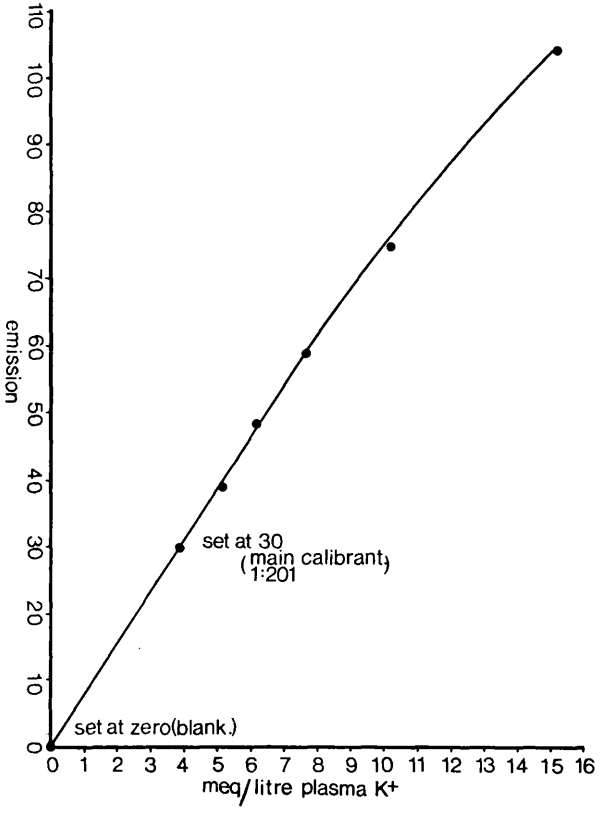


Figure 2. Calibration curve for iso-propanol diluent with cesium and hydrogen-air flame

organo-aqueous solution was used to prepare the diluent. The simplest to use was found to be iso-propanol (propan-2-ol) [4], and, providing precautions were taken to prevent evaporation, proved very reliable. A 60% alcohol-deionised water solution of iso-propanol gave a two-fold increase in emission (Fig. 1) and sample consumption was reduced to a half of that of an aqueous solution. The iso-propanol diluent was used in the same manner as the ordinary diluent. Proteins did sometimes precipitate but did not cause blockages.

The accuracy of the method was checked against a freeze dried serum solution [5] of known potassium content and was found to be within 1% of the manufacturer's figures. Reproducibility was good ($\pm$ 0.5 scale division) and the diluent remained perfectly stable.

K analysis dilution 1 : 201

Follows ZEISS handbook except:

Cesium alcohol diluent

Using MERCK ampoule 9979

1. To the contents of the ampoule in a 1 litre flask add 1 g cesium chloride.
2. Make up to 1 litre with a 60% v. v. solution of propan-2-ol in deionised water. Store in a tightly stoppered polyethylene bottle.

Calibration solution

Principal calibration solution diluted 1 : 201 with cesium-alcohol diluent.

Blank solution

Cesium-alcohol diluent.

Sample solution

5 µl of plasma diluted with cesium-alcohol diluent 1 : 201.

Flame

Air: 70% of maximum flow at FA 2.

Hydrogen: maximum flow at 4.9 lbs per square inch (.34 kgf/cm²). Valve at FA 2 fully open.

Optimum gas supply varies with the instrument, the above is a guide. The atomiser intake tubing may also be adjusted to give a sample consumption of 1 ml per minute.

Adjust wave length to 768 mµ and set slit width to .14 mm, switch on red sensitive detector and amplification 10/10/1. Stray light filter $>$ 620 mµ.

1. Atomise calibration solution and set to 30 scale divisons with amplification.
2. Atomise blank solution and set to zero with zero adjustment.
3. Atomise calibration solution and set precisely to 30 scale divisions.
4. Atomise a little diluent solution.
5. Atomise sample solution and determine concentration by means of the straight calibration line.
6. Atomise a little diluent solution (to keep atomiser clear).

References

BURRIEL-MARTI, F., and J. RAMIREZ-MUNOZ: Flame photometry. Elsevier publishing Company, Amsterdam 1957.

DEAN, J. A.: Flame photometry. McGraw-Hill, New York 1960.

ZEISS: Flame photometric serum analysis. ZEISS technical handbook GA 50—657/8/Flaz/Med/II-e.

[1] E. MERCK AG, Darmstadt, West Germany.

[2] HAWKSLY & SONS Ltd., Peter Road, Lancing, Sussex, England.

[3] EVANS MEDICAL Ltd., Speke, Liverpool, England.

[4] „Analar" reagents, BRITISH DRUG HOUSES, CHEMICALS Ltd., Poole, England.

[5] Chemical Control Serum, WELLCOME REAGENTS Ltd., Beckenham, Kent, England.

(Received February 2, 1970)

Address of the author: G. T. MAYBANK, Department of Biology, University of Stirling, Stirling, Scotland.