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STUDIES on CALCIUM METABOLISM in the RABBIT.*

by

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I N D E X.

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

Since the appearance of the works of WEISKE (1871-1874), VOIT (1901), FORSTER (1873-1876) and others of later years, numerous attempts have been made to determine the various factors involved in calcium metabolism. PATTERSON (1907), who carried out feeding experiments both on himself and on rabbits, found that a dietary deficient in calcium induced no loss of calcium in the blood, and that with a fixed diet, the urinary calcium varied but slightly, the variations being parallel with total amounts of urine excreted. One of his conclusions was that the greater the volume of urine excreted the greater was the mass of salts eliminated.

About the same time BLAIR BELL (1907), discovered a method of ascertaining the amount of calcium in the blood. He precipitated the calcium in very small amounts of blood as calcium oxalate and made a microscopic count of the crystals. He thus obtained an'index' of the amount of calcium present. This method is certainly not as accurate or as trustworthy as determining quantitatively the amount of calcium in a given volume of blood or blood serum. The work, however, was one of relative accuracy for in obtaining 'indices' at various periods, he was able to

make/

make a comparison of the amount of calcium present in the blood at these times. Following upon this, then BELL & HICK, (1909), in their observations on the physiology of the female genital organs came to the conclusion that calcium played a very important part in the caus tion of parturition. BELL'S previous findings suggested that just before birth the calcium of the mother's blood increased, but fell immediately after confinement. BELL & HICK accordingly promulgated the hypothesis that the increase in blood calcium in the later months of pregnancy is the normal stimulus for the induction of labour.

The findings of KASTLE & HEALY (1912) seemed to confirm this hypothesis. They introduced calcium peritoneally into pregnant guinea-pigs, and as a result almost invariably they got the animals to abort. According to these workers the theory of BELL & HICK was fully confirmed. More recently WIDDOWS (1923, 1924) also working on the calcium content of the blood during pregnancy, obtained results which fully supported BELL'S hypothesis, the calcium of the mother's blood rising just before birth and falling immediately after confinement.

UNDERHILL & DIMICK (1923) on the other hand failed to find evidence in support of the theory of BELL & HICK, since their figures either fell within normal/

normal limits or else blood calcium was lower rather than higher during the later months of pregnancy. They worked with whole blood believing that the corpuscles held an appreciable amount of calcium. Their findings led them to conclude that estimated upon whole blood average figures for calcium content of the blood of pregnant women are somewhat higher than non-pregnant ones. With the course of pregnancy they observed little change and therefore they could not support BELL & HICK'S contention that an increase in calcium of the blood at term was of direct significance in the induction of labour. They maintain that in general the inorganic constituents of the blood show little change during pregnancy. Moreover, according to HARDING (1925) in his review of the subject of metabolism in pregnancy, as far as blood calcium is concerned, only BELL & HICK and LAMARS had found an increase in calcium of whole blood, but some had found a slight decrease in serum in the later months of pregnancy.

Looking through the literature it was found that relatively little work had been done on calcium metabolism during pregnancy. Comparatively little had been done with regard to serum-calcium of the rabbit/

rabbit to verify or contradict the theory of BELL & HICK. Various workers had preferred to work on whole blood, accepting the view that the corpuscles held an appreciable amount of calcium. Others, again, perhaps more recent workers, had found that the corpuscles held a negligible amount of calcium, and, therefore, worked with serum. The theory of BELL & HICK seemed so interesting that the writer decided to follow up the study of calcium metabolism in the rabbit.

Since calcium metabolism plays such an important part in pregnancy, one is led to ask if calcium plays an equally important part in the reproductive life of the male as it does in that of the female and whether there is sex-dimorphism in calcium metabolism. Several years ago REACH (1912) made a comparison of the amount of calcium that could be recovered from ashing the entire bodies of male and female white mice. He worked with normal and gonadectomised males and females and obtained higher percentages of CaO from both groups of females (1·283 normal; 1·275 castrated) and lower percentages from both groups of males (1·180 and 1·005). REACH accordingly arrived at the conclusion that - "here we have a secondary sexual character - the females of these animals are richer in Ca then/

than the males". In later years HAMMETT (1923) working on the femur and humerus of rats earlier thyroparathyroidectomised at 100 days, found the amount of calcium in the males unchanged but slightly less than normal in the females, again suggesting some difference in the calcium metabolism of the sexes.

That there is sex-dimorphism in calcium metabolism has been shown by RIDDLE & HONEYWELL (1925) in their study of calcium metabolism in the pigeon. They worked on blood serum employing the method of KRAMER & TISDALL, and obtained two determinations from each bird at intervals of ten days. They obtained sex-dimorphism, the males as a group showing a lower and the females a higher calcium-content. They state, however, that only on a basis of sex had they found a consistent grouping of values.

KLISIECKI (1926) taking up the study of the difference in composition of male and female blood, found that in normal women the value for urea-nitrogen runs from 6.24 to 10.05 mg. per 100 cc. and in normal men from 10.8 to 17.9 mg. per 100 cc. thus showing that there is sex-dimorphism as far as the blood/

blood is concerned, for he obtained similar results in animals as he did in man. Another instance of sex-dimorphism was shown by CHAUDHURI (1926) who, working in these laboratories on the erythrocyte count in sexually normal fowls, found definite sex-dimorphism, the number of erythrocytes in a unit volume of blood being significantly higher in the adult male than in the adult female. His work is in general agreement with the findings of BLACHER, whom he quotes. CHAUDHURI points out in his paper that for sex-dimorphism the birds must be sexually mature and normal, because he found no difference in the erythrocyte count in sexually immature male and female fowls. Now, if there is such definite sex dimorphism in the erythrocyte count, and if the erythrocyte holds an appreciable amount of calcium, then working with blood one should obtain sex-dimorphism in calcium-metabolism. If one obtains a difference in whole blood, it would be very interesting to find sex-dimorphism in the calcium of blood serum.

It is not so long ago since MANOILOV (1922 - 1923) originated what is now known as the MANOILOV'S reaction. He was able to distinguish the sexes by applying certain chemical reagents. Since his work on/

on plants, his reaction has been applied to tissues and blood of mammals to distinguish the sexes. Some workers are confident of the MANOILOV reaction but others are not. ALSTERBERG & HAKANSSON (1926) state that only oxidising and reducing substances play any part in MANOILOV'S reaction, and that they could trace no relation between sex and this reaction. CREW (1927) in his paragraph on sex-dimorphism refers to GRÄFENBERG (1922), MANOILOV (1922-23), SATINA & DEMEREC (1925) and EDLBACHER & RÔTHLER (1925) who have recently described certain pieces of experimentation that bear directly upon this question of sex dimorphism, for they confirm the impression that the sexes are biochemically distinct.

On examining the literature one is somewhat convinced that the sexes are biochemically distinct. CHAUDHURI'S work on the erythròcyte count has shown that sex-dimorphism in a physiological sense is very evident. At the same time the distinction between the sexes may be so insignificant that it is noticeable only at certain periods. RIDDLE & REINHART (1926), in their work on calcium metabolism in the pigeon and ring dove, found a fairly constant value for the males. This value was the same for the females except at ovulation, when there was a great increase in calcium.

These/

These workers accordingly state that there is no sex-difference in the resting periods, but there is very evident sex-dimorphism at the ovulation periods. If such is the case in the pigeon, it would be very interesting to find sex-dimorphism in the calcium metabolism in rabbits. With this object in view the writer decided to continue his experiments on rabbits of both sexes, and, having collected data, to make a comparison of the values for the sexes.

A continuation of the study of calcium metabolism in rabbits leads one to consider what relationship there may be between the serum-calcium content of the mother and her offspring. An examination of the literature shows that FENGER (1912) made comparison of the iodine content of desiccated, fat-free glands of the ewe and foetus and of the sow and foetus. In every case the glands of the mother showed a higher percentage of iodine. Since then comparatively little work has been done on the calcium content of maternal and foetal blood or serum, and that which has been done is more or less recent. This line of research, moreover, has been followed up mostly in cattle and in the human. ROBINSON & HUFFMAN (1926) found that the blood of the mother at calving had a lower calcium content than that of the calf, and that the tendency in the case of the calf was to decrease during the first week. In the case of the mother there was a drop in calcium at the close of pregnancy, but after calving there was an immediate rise, which frequently was not completed for several days. HELLMUTH (1925), working on the human, found that the calcium in the foetal blood was always 20-30% higher than in the maternal blood. H. A. & H.I. MATTIL (1922), also working on the human, found on the other hand that at birth infant's and mother's blood were the same in calcium content, and during several months after birth, the calcium in the infant's blood increased, reaching a variable maximum, often double that at birth. Thereafter there was a gradual decrease in the infant's blood. HARDING (1925), in his review of metabolism during pregnancy, shows that Ca, P, Na, and K appear to be higher in foetal than in maternal blood. These various findings in blood calcium indicate that foetal blood has more calcium than maternal. ROBINSON & HUFFMAN have shown that at the close of pregnancy there was a drop in calcium in the maternal blood, followed by a rise immediately after calving. Now if the calcium level is maintained in the foetal blood, at calving, it is reasonable to presume, there would be more calcium in foetal than in maternal blood as was found by HELLMUTH. But H.A. & H.I. MATTIL/

MATTIL found that the calcium in the foetal blood equalled that in the maternal blood. These findings are somewhat conflicting, so it was thought advisable to group together the data for the mother rabbits, obtained so far, and compare them with data obtained from their litters. In this way it seems possible to make a comparison in the calcium of maternal and foetal blood.

MATERIAL/

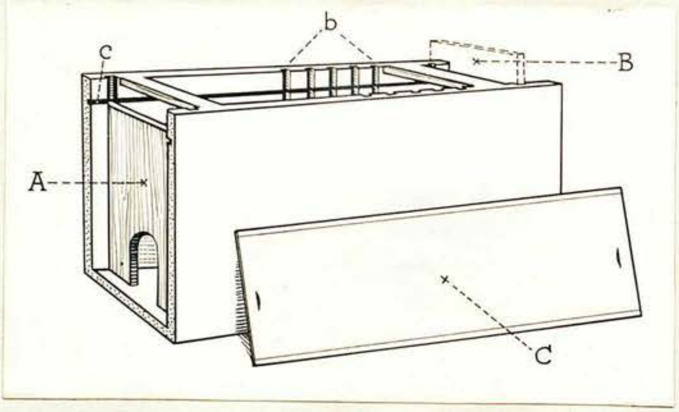


FIG. 1. BLEEDING BOX.

FIG. 2. BLEEDING BOX with RABBIT in POSITION.

M A T E R I A L .

Rabbits were found to be the most convenient animals to work with, for it was necessary to obtain samples of blood frequently and for a period of time from the same animal. Choosing a rabbit with fairly large ears, it was found possible to obtain the necessary samples of blood from the ear veins as frequently as the experiments required them. The drawing of the blood was done usually every third day, but this in no way weakened the animal or upset its metabolism. Provided the animal was adequately fed and looked after, there was no difficulty in obtaining the required amount of blood from it. The bleeding was done very conveniently by placing the animal in a wooden box, which was long and broad enough to hold the larger size of rabbits. (See FIGS. 1 & 2.) The space for the rabbit could be adjusted to suit the length of the animal by moving the back end B., i.e. by lifting it up and placing it into the vertical slots or grooves b., on the sides of the box. The front end A., of the box was fashioned out at its bottom to accommodate the neck of the rabbit, and this like the back end also fitted into two vertical grooves. The lid of the box C., was

so/

so fashioned that it slid into two horizontal grooves c. on the sides of the box, and on being moved into position it closed in both the front and back ends of the box, as well as the body of the rabbit, leaving only the head of the rabbit projecting out at the front.

With the rabbit held in this position bleeding was easily done by making a small incision with the end of a razor over one of the ear-veins, and collecting the blood into a small tube. Shaving, washing or disinfecting the seat of operation was unnecessary, provided the ear was clean and not too abundantly covered with hair. If the vein did happen to be hidden by a too thick growth of hair, it was best to shear off the hair with a pair of scissors. The blood was collected into a small flat-bottomed tube measuring 5·5 cm. x 1·8 cm. with the walls 0·5 mm. in thickness. It was found less convenient to catch the blood if the walls were much thicker than 0·5 mm. The tube was graduated on the outside in 2 cc. so that there was no difficulty in knowing when one had collected 4 cc., the required amount to give the volume of serum necessary for analysis. For the sake of convenience, it was best to have a tube for each rabbit,/

rabbit, numbering it the same as the number of the rabbit.

Having collected 4 cc. blood, the haemorrhage was stopped by digital pressure, wiping away the blood from around the incision and applying a drop of collodium. When this collodium set, it formed a skin over the incision and prevented haemorrhage, if the rabbit scratched its ears. For the next sample of blood from the same animal, the incision was made a few mm. below the last one, and in this way it was possible to work down the length of the vein from its wider end to its finer branches. After collecting 4 cc. the tube was corked up and put aside for the blood to coagulate over night. Normally the blood coagulated quite satisfactorily over night, but temperature had a marked effect on the degree of coagulation, in so much that during summer 4 cc. blood gave at least 2 cc. serum, but during winter, unless the room had been kept warm during the whole day, the same volume of blood gave less than 2 cc. serum. The right amount of serum was obtained, however, by heating the tubes for a few minutes over a radiator, a few hours before the serum would be required, and then cooling them to room temperature by placing in cold water.

During the warmer weather it was an easy matter collecting 4 cc. blood from each rabbit,

but/

but in colder weather the blood did not flow so freely, and often it was a difficult matter obtaining the 4 cc. Friction with the fingers over the vein sometimes enabled the blood to continue its flow, but it was found best to transfer the rabbit in a basket to the top of a radiator. After a few minutes the ears became warmed up and bleeding could be done as easily as on a warm day. By this means it was found possible to bleed the rabbits even on the coldest days.

It was an easy matter bleeding the adult or almost adult rabbits, as described above, but it was practically impossible to bleed the young litters in the same way. Until the young rabbits were about a month old, it was out of the question placing the sucklings into the bleeding box, for the purpose of obtaining blood from them. With the larger breed of rabbits, when the young rabbit was a month old, it was possible to place it in the bleeding box, and the blood was obtained as described. With the rabbits younger than a month, it was found best to obtain samples of blood by decapitation. Until the rabbits were two to three weeks old, it was not possible to obtain 4 cc. blood by decapitation, but usually enough/

enough blood could be collected to give 1 cc. serum. The blood of the litters taken out by laparotomy was also obtained by decapitation. In this latter case each foetus did not yield even 2 cc. blood, but enough blood was collected from all the foetuses removed, to give at least 2 cc. serum, the result serving as an average for the whole litter.

From the results of previous investigators it would seem that the ordinary food of the rabbits would not make differences in their calcium metabolism. The rabbits used in the experiments were fed and attended to by the writer. The foods given were the ordinary rabbit foods - oats, bran, flaked maize, wheat, hay, cabbage leaves and other green stuff in the form of clover hay. When the rabbits were given green feeding in the form of cabbage leaves, and fresh green grass they did not need water, but failing green stuff, they were watered twice a day. It seems justifiable to assume that the food and daily care and attention to the rabbits would make no variations in the calcium findings. Experience showed that the animals were not to be handled harshly or excited unnecessarily, as this assuredly led to a fall/

fall in the serum calcium which was not expected. Although the ordinary food of the rabbits would not tend to alter the calcium findings in the serum to any great extent, in the case of the urine, however, there seems to be a loss of calcium in proportion to the volume excreted. For this reason it was considered best to work with venous blood, which could be obtained quite easily from the ear veins.

TECHNIQUE/

T E C H N I Q U E .

The method adopted for the whole series of analyses was that of KRAMER & TISDALL (1921) with slight modifications. As a rule the serum of four rabbits was worked with each morning, since the centrifuge held no more than four tubes. Four 2 cc. pipettes were used for delivering the serum, and these being marked I-IV., there was a separate pipette for each tube. The same four animals were bled in the same order every third day, so that the serum of the same animal was always delivered with the same pipette, thus eliminating any inaccuracies there might be due to slight differences in volume of the 2 cc. pipettes. In the analysis the end point was obtained after titration with a 2 cc. microburette. On testing,this microburette was found to be accurate. Before using the 2 cc. pipettes, the volume of each was compared with the volume of the microburette, and those proving inaccurate were rejected. So it may be assumed at this point, that the 2 cc. pipettes used for analysis were accurate as compared with the microburette. The centrifuge tubes used were small and conical and were/

were graduated to hold 10 cc.

The routine and technique used for the analyses were:-

2 cc. sat.ammonium oxalate was poured into each of four centrifuge tubes. Using the necessary pipette for the serum of the rabbit of the same number, 2 cc. serum was dropped into the oxalate, and the pipette left in a slanting position for the serum clinging to the sides to drain to the tip. In the meantime the centrifuge tube was shaken in a circular motion, great care being taken not to splash the slightest drop of liquid out of the tube. The pipette was then completely drained into the tube which was again shaken up till all the particles of precipitate were seen to be disturbed. After the four centrifuge tubes were so treated, the inside of each tube was washed down with 2 cc. distilled water, delivered with a 2 cc. pipette kept for that purpose only. The four tubes were then allowed to stand for 30 minutes, allowing time for all the calcium to be precipitated.

The tubes were then centrifuged in an electric centrifuge at full speed (about 3,000 revs.per minute) for 5 to 10 minutes. If the centrifuge is in proper functioning order, this time is sufficient for all the precipitate to be separated. The supernatant/

supernatant fluid was removed by decantation, by gently sloping each tube, and then leaving the tube in a slanting position to drain off all the liquid. The test tube holder was modified to hold these tubes both upright and in a slanting position. After five minutes, the mouth of the tube was dried with a clean duster, and 2 cc. 2% ammonium hydroxide was poured in, after which the precipitate at the bottom of the tube was thoroughly shaken up in the liquid. The inside of the tube was then washed down with 2cc 2% NH_4OH. Having treated the four centrifuge tubes thus, they were centrifuged at maximum speed for 5 minutes, the supernatant liquid decanted off, and the tube left in a slanting position for as long as possible, to drain off the liquid. The mouth of the tube was then dried, and the precipitate was ready to be dissolved and titrated. The method of KRAMER & TISDALL demands that the precipitate should be washed three times to free it from magnesium, but a publication that year of CLARK & COLLIP (1925) and personal experience showed that one washing of the precipitate with 2% NH_4OH was sufficient to free it from magnesium.

Having dried the mouth of the tube 2 cc.approx. $N.H_2SO_4$ was poured in and the precipitate dissolved by/

by heating the tube in a boiling water bath for a few minutes. While still hot the liquid was titrated as quickly as possible with a weak solution of potassium permanganate delivered from a 2 cc. microburette graduated in 0·01 cc. The titration was done drop-wise to lessen the chances of going over the mark, the end point being taken when the pink colour lasted for at least 1 minute. The calculation was done from the formula:-

$$\frac{xcc\ KMnO_4 \times 10 \times y}{\cdot 01} = \text{mgm.Ca per 100 cc.serum}$$

where x = volume of $KMnO_4$ used

and y = normality of $KMnO_4$

y was found by titrating the $KMnO_4$ against N/100 $Na_2C_2O_4$, using the formula:

$$\frac{10cc \cdot 01N\ Na_2C_2O_4 \times \cdot 01}{x\ cc\ KMnO_4} = y.$$

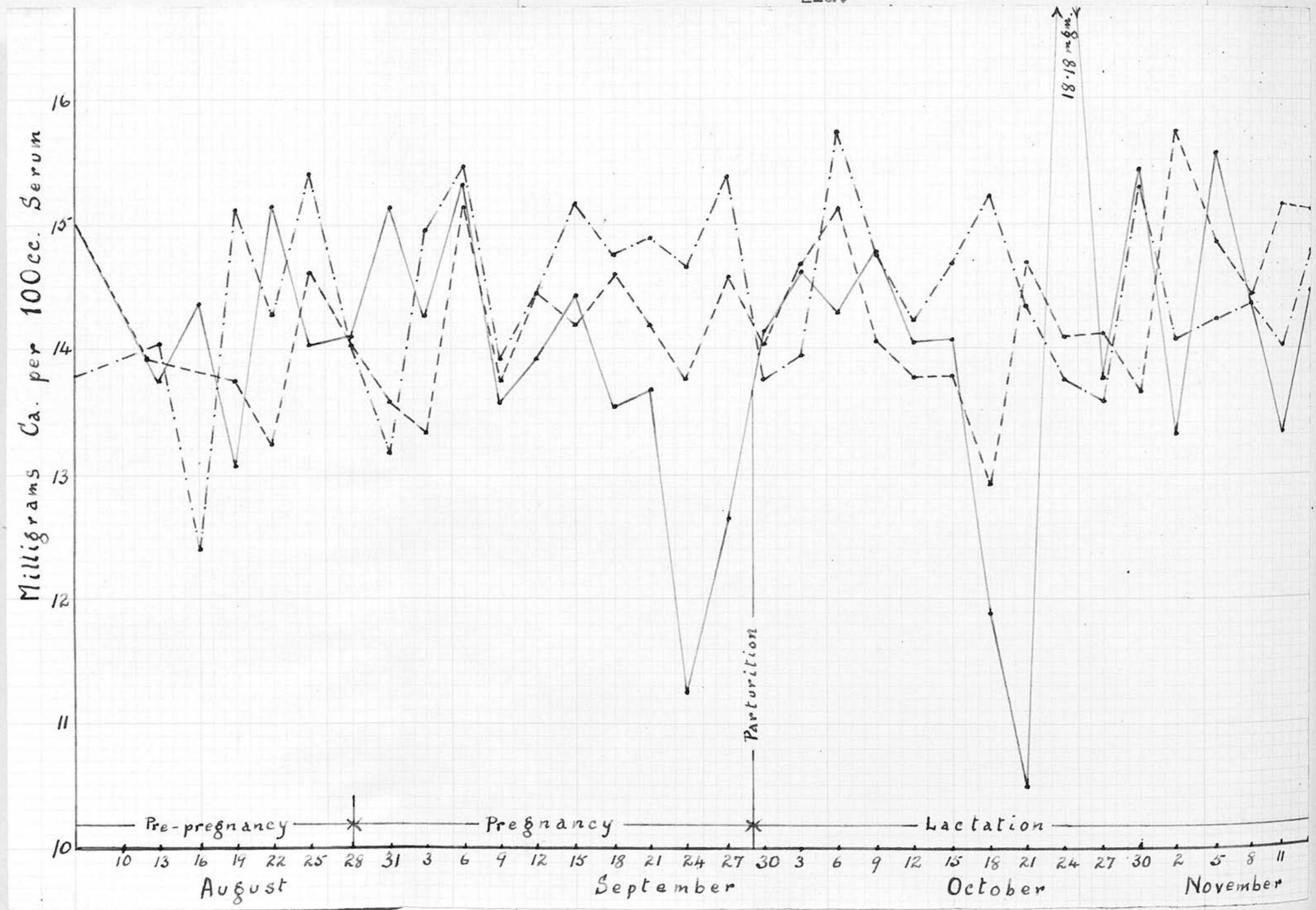
10 cc N/100 $Na_2C_2O_4$ being titrated against the $KMnO_4$.

The permanganate solution was made up according to HALVERSON & BERGEIM (1917) but the 0·0133N $KMnO_4$ was diluted still further to make the solution in the neighbourhood of 0·0117N. The strength of this $KMnO_4$ solution was determined every day by titrating against N/100 $Na_2C_2O_4$ (Sörensen), made up as described by KRAMER and TISDALL (1921).

I./

I. CALCIUM METABOLISM in the PREGNANT and LACTATING RABBIT.

The experiment was started on three Himalayan virginal rabbits, which were about six months old. Blood was taken from each at three-day intervals for about a month before they were mated. In this period the calcium in their blood serum varied as much as 13 to 15 mgm. per 100 cc. serum, the drop or rise sometimes being as much as 2 mgm. at the end of three days. This showed that there was variation, but the variation was not taken as being abnormal, because preliminary work that had been done showed as much variation. Miscellaneous rabbits had been bled previously for a period of a few weeks, and their variations appeared as great as those exhibited by the Himalayan rabbits. Consequently the variations shown by these latter rabbits were not accepted as being abnormal. The variations, moreover, in these Himalayan rabbits appeared to be more or less regular, i.e. one day the finding was 15.05 mgm.Ca; three days later the value had fallen to 13.76 mgm; six days later there was a rise to 14.37; nine days later there was a fall to 13.07 mgm; while twelve days later there was a rise to 15.14 mgm. There was somewhat of a regular/



GRAPH I. —— Himalayan ♀A. Mated 28/8/26 Littered 29/9/26
---- " ♀B " 27/8/26 Aborted
-.-. " ♀C " 27/8/26 "

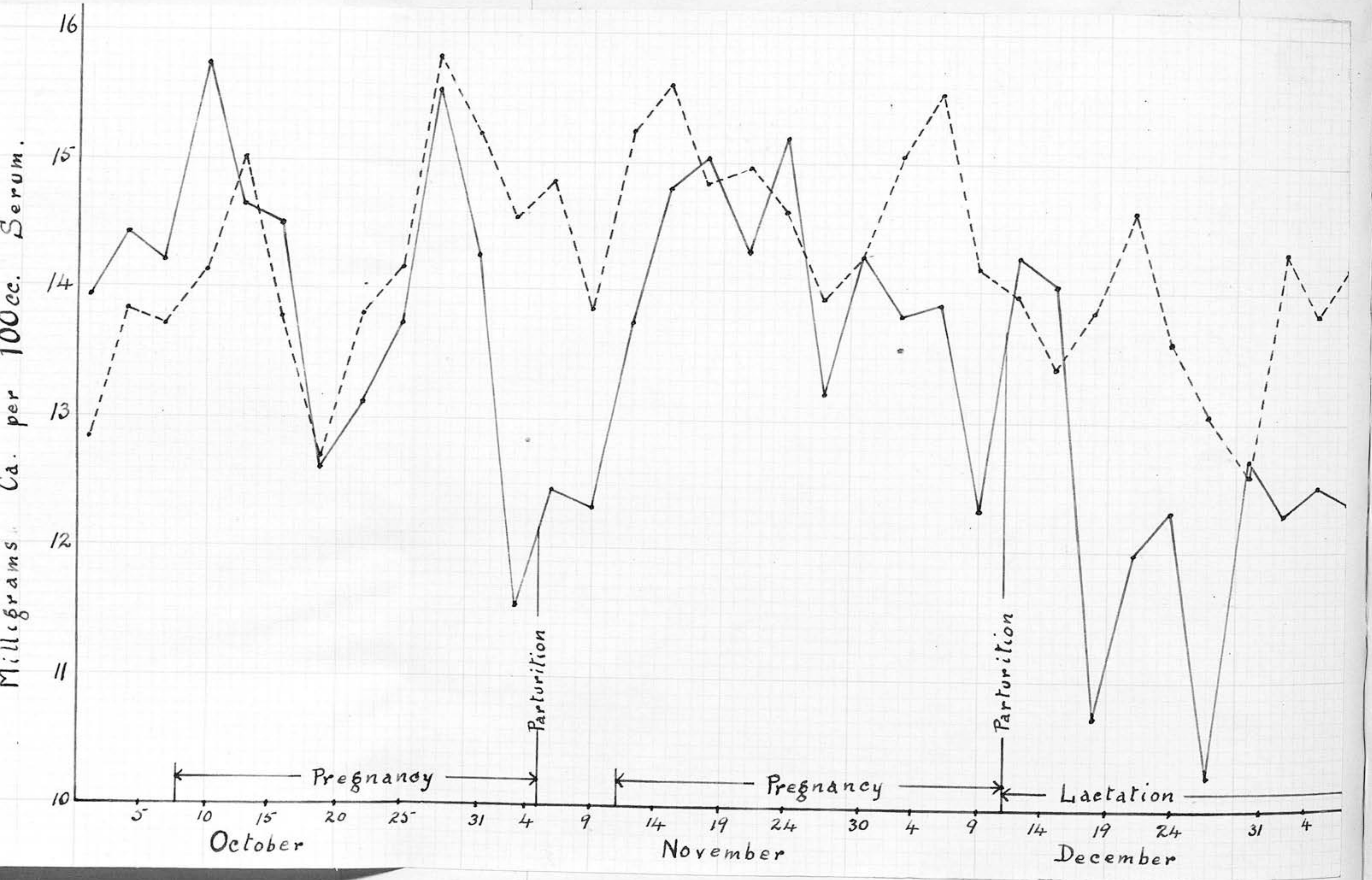
regular rise and fall.

At the end of practically a month these three Himalayan does were mated, and the bleeding was continued at 3-day intervals, assuming that they were all pregnant. Rather than submit columns of figures, it was thought best to submit the results in the form of graphs. The results obtained from these three Himalayan does are shown in Graph I., which shows a graph of the periods of pre-pregnancy, pregnancy and lactation. Here it will be seen that in early pregnancy there is not very much difference between one pregnant rabbit and another, and there are variations as there were before pregnancy. The amount of calcium in the serum again varied roughly between 15 and 13 mgm., and till about the middle of the period of pregnancy, it is difficult to distinguish between one doe and another. It will be seen that on the 18th day of pregnancy, the values for ♀A ♀B and ♀C were 14·43 mgm., 14·19 mgm., and 15·17 mgm. respectively. Three days later the value for ♀A had fallen slightly below those of ♀B and ♀C, the values being 13·52, 14·59 & 14·76 mgm. respectively. Six days after this, i.e. on the 27th day of pregnancy, the values of ♀B and ♀C were 13·76 mgm. and 14·66 mgm. while that of ♀A had fallen suddenly to 11·26 mgm.

Three/

Three days later the value for ♀A rose but was still lower than those of ♀B and ♀C, the values being 12·64 mgm, 14·56 mgm. and 15·38 mgm. respectively. Two days after this ♀A littered, i.e. the minimum for the period of pregnancy was reached five days prior to parturition. It will be noticed from the graphs that only ♀A showed this sudden drop in the serum calcium, and moreover, she was the only rabbit that littered, and entered into a period of lactation. During the early period of lactation there is not much difference between the three rabbits, but at the end of the second week of lactation ♀A showed a sudden drop in her serum-calcium to 11.9 mgm. Three days later there was a still further drop to 10·5 mgm i.e. the lactation minimum. Three days after this minimum was reached, the serum-calcium soared to 18·18 mgm., three days after which the figure dropped to normal. During this period ♀B and ♀C showed no remarkable variations, the values still varying between 13 and 15 mgm. Throughout the remaining period of lactation the serum-calcium of ♀A remained normal, also varying between 13 and 15 mgm.

In the Himalayan rabbit the ear-length is rather small and, therefore, the same ear was capable/



GRAPH II. —— ♀D Mated 8/10/26 Littered (still-born) 5/11/26
"11/11/26 " 11/12/26

----- ♀H Virgin (control)

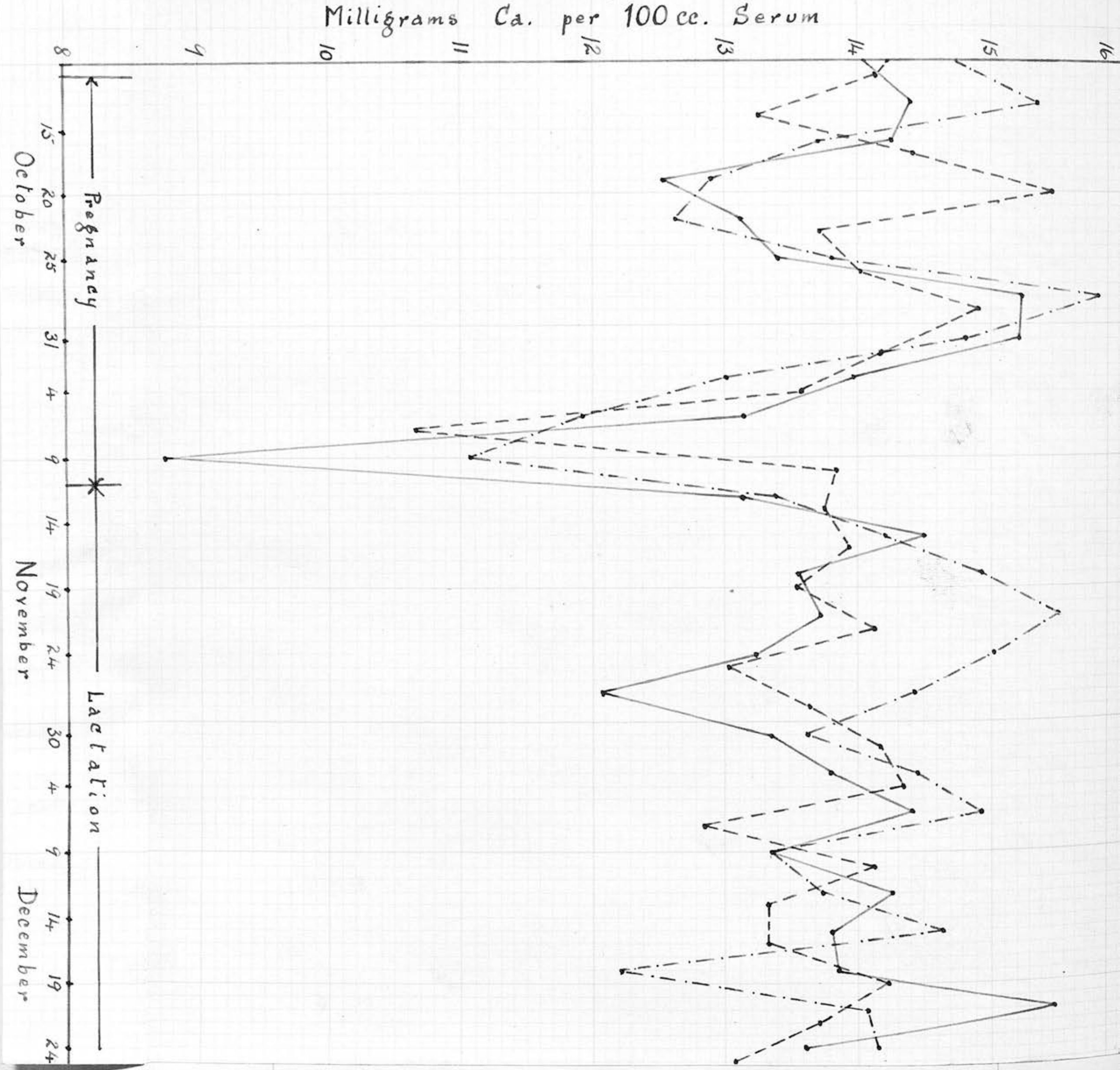
capable of a limited number of bleedings. It was found more convenient to have a larger length of ear, so that the same ear did for more numerous bleedings. The Department was fortunate in obtaining young rabbits that were crosses between the Belgian Hare and the big lop-eared Flemish Giant. These rabbits proved to be excellently suited for bleeding purposes and could be bled conveniently for at least a year at three-day intervals.

Work was started on five does of the Belgian Hare crosses ♀♀D, E, F, G & H. They were all of the same age, about five months, but ♀H appeared the smallest and was accordingly kept unmated for a while and, therefore, served as a control. These five does were not necessarily bled on the same day, but all were bled at intervals of three days.

Graph II. shows a graph of the figures for each of the does ♀D and ♀H, and as ♀H was kept unmated, the graphs show whatever differences there may be between a pregnant and a virgin doe. Till just before parturition it will be again noticed there is practically no difference between the figures for one doe and another. Two days before parturition, however, the graph of ♀D shows a sudden fall to the pregnancy minimum. Eight days before parturition the figure/

figure for ♀D was high, 15·58 mgm., but three days after this there was a fall to 14·27 mgm, three days after which was the sudden fall to the pregnancy minimum of 11·55 mgm. At this point we may say there is a distinct difference between the pregnant and the non-pregnant rabbits. This difference, however, is very transitory, because a week after parturition, the figure for the once pregnant rabbit rises suddenly to normal, 13·78 mgm., 14·8 mgm. etc.

The litter of ♀D was still born, so that no lactation period followed. ♀D was, therefore, mated up again soon after, but unfortunately she was allowed to remain unmated for too short a time to make a true comparison between the two does. Even after ♀D was remated and made pregnant for the second time, there appeared to be little or no difference between the figures for the two does, till towards the close of pregnancy. Again, 8 days before parturition the serum-calcium of ♀D showed signs of declining. Two days before parturition the fall was again sudden, though not so great to the pregnancy minimum of 12·34 mgm. but three days after parturition the figure soared to normal, 14·28 mgm. Early in the lactation period/



GRAPH III. -.-. ♀E Mated 11/10/26 Littered 10/11/26

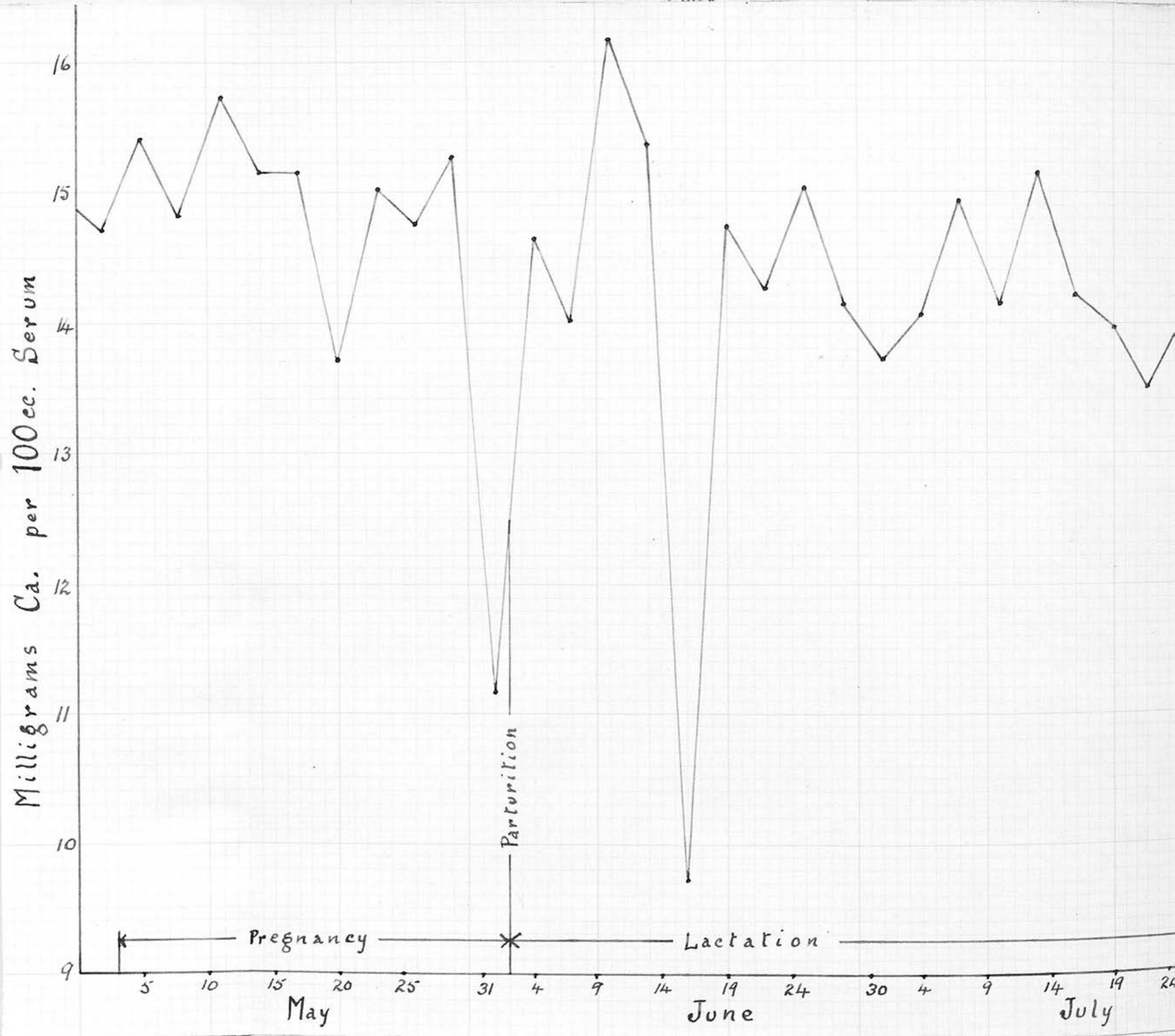
—— ♀F " 11/10/26 " 11/11/26

---- ♀G " 9/10/26 " 9/11/26

period, there seems to be no difference between the two does, but at the end of the first and second weeks of lactation, respectively, there is a fall in the serum-calcium of ♀D, and a minimum is reached which is much lower than the pregnancy minimum, two minima 10·75 mgm. and 10·27 mgm. being recorded. During this period the figures for ♀H, it will be noticed, are between 13 and 15 mgm.

Graph III shows graphs for the figures of ♀♀E, F, and G. In early pregnancy there is some slight variation in the figures for the same animal, but there is not much difference between one animal and another. Soon after the mid-pregnant period is reached, there seems to be a rapid rise in the serum-calcium, shown by all three does. The maximum seems to be reached by the 10th day before parturition, after which there is a rapid, and in the case of ♀F a sudden fall to the lowest pregnancy minimum of the three does. About the tenth day before parturition, the figures for the three does were 15·91 mgm 15·25 mgm. and 14·92 mgm. respectively. Two days before parturition ♀G had fallen to 10·67 mgm., ♀F to 8·74 mgm. and a day before parturition ♀E to 11·08mgm

It/



GRAPH IV. ♀H Mated 3/5/27 Littered 2/6/27

It is interesting to note that the graphs of all the animals that were truly pregnant show often a marked increase in the serum-calcium towards the close of pregnancy, but always there is a sudden fall (1, 2, or 3 days) before parturition.

Graph IV. shows a graph of the serum-calcium of ♀H, but this time in a pregnant condition, There are slight variations in early pregnancy, but as the mid-pregnant period passes, there seems to be a fall with soon a rapid rise to over 15 mgm. Following this there are slight variations but a day before parturition there is the usual sudden fall to the pregnancy minimum of 11·17 mgm. To complete the similarity with the figures for the other rabbits, two days after parturition the serum-calcium soared to 14·65 mgm.and six days after this to 16·15 mgm., the lactation maximum.

An examination of the graphs shows that in every case of true pregnancy, followed by a period of lactation, there is a sudden rise after parturition to normal and sometimes above normal in serum-calcium. In the case of ♀A, during the first two weeks of lactation, the serum-calcium remained fairly high, i.e. in the neighbourhood of 14 mgm. Soon after/

after this there seemed to be again a sudden fall, the minimum reached being much lower than that before parturition, e.g. the pre-parturition minimum was 11·26 mgm. whereas the lactation minimum was 10·5 mgm. Three days after this minimum was reached, there was a very sudden rise to the lactation maximum of 18·18 mgm. This maximum was indeed very peculiar, for three days later the serum-calcium had fallen to normal. ♀A was the only rabbit that exhibited such an immense lactation maximum.

The graph of ♀D shows that there was a sudden rise after parturition, the figure rising to 14·28 mgm but six days after this there was a sudden fall to 10·75 mgm. Six days later the serum-calcium rose to 12·31 mgm., but at the end of nine days there was a still larger drop to the lactation minimum of 10·27mgm Thereafter, and till the end of the lactation period the serum-calcium showed a tendency to rise rather gradually, arriving at the normal of 14·17 mgm. when the young were separated from their mother.

The graph of ♀E shows that soon after parturition the serum-calcium had soared to a bove normal, reaching the high figure of 15·55mgm. in the middle of the second week of lactation. Thereafter there was a rise/

rise and fall, but the fall was never in the neighbourhood of the pre-parturition minimum. The graph of ♀F shows a fall during lactation, greater than those experienced by ♀E and ♀G. Two days before parturition her pre-parturition minimum of 8·74 mgm. was found, but a day after parturition the serum-calcium soared to 13·13 mgm., and three days after this to 14·5 mgm. As lactation progressed the serum-calcium seemed to fall, a minimum of 12·09 mgm. being reached at the beginning of the third week of lactation. Thereafter the serum-calcium seemed to rise to the neighbourhood of 14 mgm., and remain there with slight variation till the litter was weaned. The graph of ♀G shows that similar to ♀F her pre-parturition minimum of 10·67 mgm. was reached two days before parturition. On the day after parturition, her serum-calcium also soared to 13·88 mgm. Thereafter the figure seemed to remain normal though there were slight variations during lactation.

The graph of ♀H seems to be the most remarkable of these Belgian Hare crosses during the lactation period. The pre-parturition minimum, reached a day before parturition, was low, 11·17 mgm. but two days after parturition, the figure rose suddenly to 14·65 mgm. Then there was a slight fall, but eight days after/

after parturition, the abnormal maximum of 16·15 mgm was found, -. (c.f. graph ♀A). Six days after this maximum was r ecorded, the serum-calcium fell very suddenly to the lactation minimum of 9·71 mgm. The rise to normal was as sudden for three days later the serum-calcium was at 14·74 mgm. The serum-calcium thereafter seemed to remain normal in the neighbourhood of 14 mgm. It must be noted that none of the Belgian Hare cross does showed the most abnormal maximum that the Himlayan ♀A showed at the close of the third week of lactation.

CONCLUSIONS/

C O N C L U S I O N S .

The graphs of the rabbits ♀A - ♀H show that under normal conditions, there is little or no difference between the early pregnant and non-pregnant animals, the serum-calcium being in the region of 14 mgm. per 100 cc. serum. There are slight variations at the 3-day intervals, but it is in the last week or so of pregnancy that it is possible to differentiate between a pregnant and a non-pregnant rabbit. From about a week before parturition, the serum-calcium, which is then normal or slightly above normal, commenced to fall and although four days before parturition, the figure may be above normal, a day or two before parturition it declines suddenly to the minimum of the pregnant period. The figures for the non-pregnant on the other hand remain more or less normal. This sudden fall in the serum-calcium before parturition is remarkable and appears to be true in all cases of true pregnancy in the rabbit.

According to DIXON & MARSHALL (1924) there is an Ovario-Pituitary Endocrine Mechanism which comes into operation at the close of pregnancy, "when the corpora lutea are in an advanced stage of involution/

involution" the normal secretory activity is once more produced and the pituitary is excited to secrete im greater quantity", so resulting in parturition. According to these authors, then, parturition is caused by a greater secretion from the pituitary. If such is the case, the above results show that at this special time, not only is the pituitary excited to secrete but a state of acidosis in the blood suddenly sets in. The excitement of the pituitary not only causes that endocrine to secrete, but also brings about a very sudden depression in the serum-calcium, perhaps through the pituitary secretion. Whatever it is that causes this sudden depression does its work very suddenly, but the calcium metabolism of the mother regains its normality, in most cases, as suddenly.

This sudden fall in the serum-calcium prior to parturition, is very comparable to the fall experienced in rabbits that have undergone an operation, e.g. a male rabbit had a fairly high normal serum-calcium, 14 to 15 mgm., but blood collected soon after the rabbit was castrated, gave a reading of 12 mgm. calcium, a figure very much lower than was expected. This phenomenon just before parturition makes the matter appear as if the whole system were "shocked" as happens after an/

an operation. The results obtained from the preg-
nant rabbits fail to support the theory of BELL and HICK that an increase in blood calcium at the close of pregnancy is the normal stimulus for the induction of labour. The results show rather that at the close of pregnancy there is a sudden fall in blood calcium and it is very probable that a state of acidosis in the blood is the normal stimulus for the induction of labour, and not an increase in blood calcium.

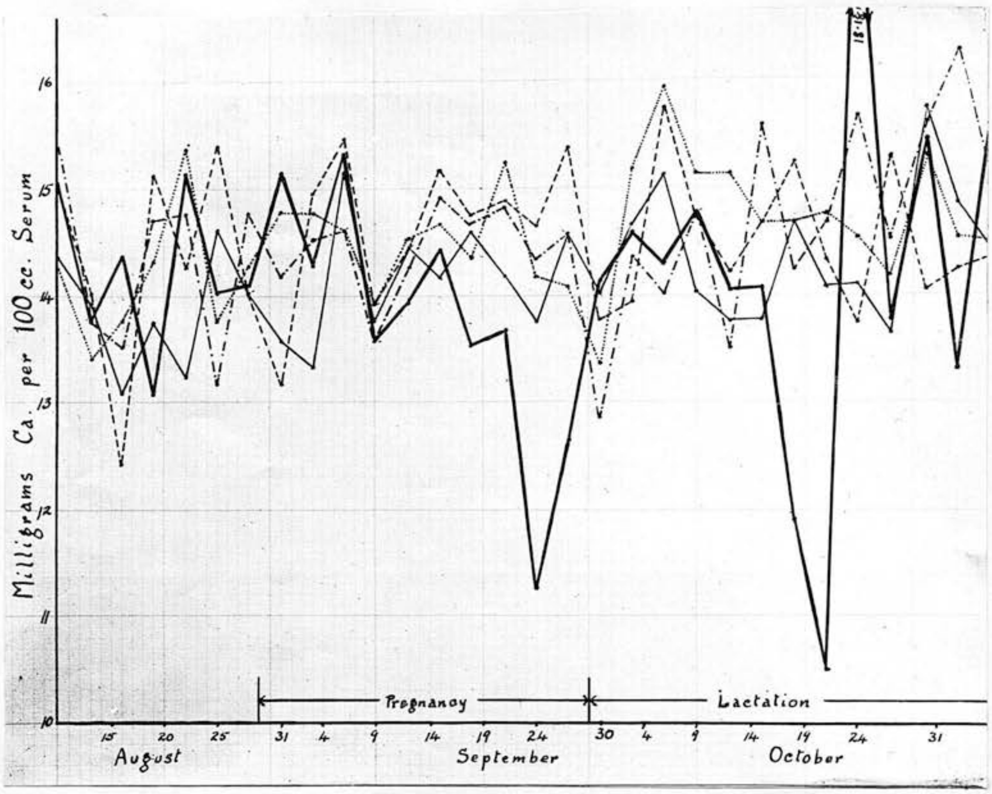
II/

II. CALCIUM METABOLISM in RELATION to SEX.

The sense of biochemical distinction between the sexes, aroused one's interest while doing work in calcium metabolism in rabbits during pregnancy and lactation. Previous to this, preliminary work had been done on various rabbits to become familiar with the technique for the estimation of calcium in blood-serum. This preliminary work gave an indication that there was little or no sex-dimorphism in calcium-metabolism in rabbits, but as time went on and a fair amount of data had been collected, there seemed a vague indication that there was sex-dimorphism, especially and very obviously during pregnancy and lactation.

This part of the work was done on 30 rabbits of both sexes. Some of them were sexually mature, but the others immature when bleeding was started on them. The blood was obtained as described previously, and the calcium was estimated by the modified method of KRAMER & TISDALL.

The animals used first were two male and three female Himalayan rabbits, members of the same litter and about six months old when bleeding was started./



GRAPH V. ——— Himalayan ♀A Mated 28/8/26 Littered 29/9/26

—— " ♀B " 27/8/26 Aborted

---- " ♀C " 27/8/26 "

-.-. " ♂A

.... " ♂B

started. The data collected do not include periods of pregnancy and lactation i.e. from the time of mating till the weaning of the litter. The accompanying graph (Graph V.) gives an idea of the actual figures obtained for a period of two months. As noted on the graph, the females were mated, but only one, ♀A, became pregnant and littered. An examination of the graph shows that only in the case of ♀A do we see any signs of sex-dimorphism, e.g. when after 20 days of pregnancy there is a sharp and sudden decline to a minimum found 5 days before parturition. When, however, we do not consider the graph of ♀A and examine the graphs of her brothers and sisters, it is questionable if we can distinguish the males from the females. There seems to be a similar rise and fall in calcium in both sexes. ♀B and ♀C were mated but they did not become pregnant, and this is perhaps the reason why their graphs resemble more those of the males than that of ♀A. The only conclusion we can come to from the graphs is, that except during pregnancy, there are no grounds for saying that there is sex-dimorphism.

Now, however, if the data for these Himalayan rabbits, excluding those for the periods of pregnancy and lactation, are subjected to treatment and the true mean with probable error found as suggested in FISHER'S book on statistical methods we have:-

RABBIT	OBSERVATIONS	HIGHEST	LOWEST	TRUE MEAN with PROBABLE ERROR
♂A	35	15.58	12.87	14.71 ± .089
♂B	35	15.93	13.36	14.69 ± .085
♀A	28	15.56	11.77	13.89 ± .109
♀B	18	15.14	13.09	14.11 ± .105
♀C	18	15.48	12.41	14.33 ± .13
♂♂A,B	70	15.93	12.87	14.7 ± .061
♀♀A,B,C	64	15.56	11.77	14.17 ± .068

According/

According to the data there are slight differences between the males and between the females. It was, however, thought best to consider all the observations of each sex together and so find the true mean with probable error for each sex. The estimations then for the sexes are:-

$$♂♂\ 14.7 \pm .061$$

$$♀♀\ 14.17 \pm .068$$

$$\therefore \text{Difference} = .53 \pm \sqrt{.061^2 + .068^2} = .53 \pm .091$$

∴ the difference is 5.82 times its probable error (.091).

The data for the other adult rabbits when computed are:-

RABBIT	OBSERVATIONS	HIGHEST	LOWEST	TRUE MEAN with PROBABLE ERROR
♀D	31	15.97	13.37	14.42 ± .107
♀E	36	15.85	11.91	14.0 ± .112
♀F	36	15.51	12.08	13.97 ± .101
♀G	36	16.08	12.3	13.97 ± .101
♀H	36	15.83	12.31	14.22 ± .099
♂C	36	15.94	12.96	14.36 ± .088
♂D	36	15.75	12.56	14.47 ± .081
♂E	36	16.42	12.83	14.53 ± .077
♂♂C,D,E	108	16.42	12.56	14.45 ± .048
♀♀D - H	175	16.08	11.91	14.11 ± .047

The estimations for the sexes then are:-

♂♂ 14.45 ± .048

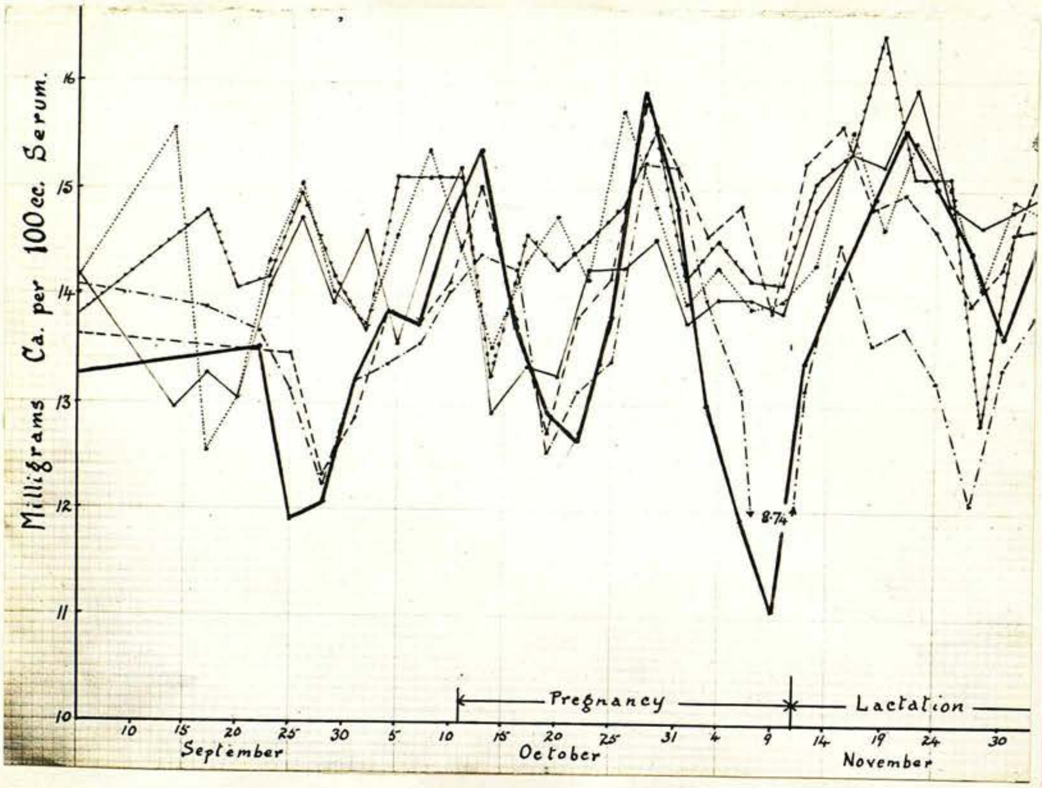
♀♀ 14.11 ± .047

$\therefore$ Difference $= .34 \pm \sqrt{.048^2 + .047^2}$

$= .34 \pm .067$

$\therefore$ the difference between the sex values is 5.07 times its probable error (.067).

Graph/



GRAPH VI

———	♀E	Mated	11/10/26	Littered 10/11/26
-.-.	♀F	"	11/10/26	" 11/11/26
----	♀H	Virgin (control)		
———	♂C	Castrated	14/9/26	
.....	♂D	Entire, normal.		
—•—•—	♂E	Vasectomised	26/11/26	

Graph VI shows a graph of the actual figures for each of these rabbits with the exception of ♀D and ♀G. As stated ♀H was kept virgin as a control, while ♀E and ♀F were mated up. As in Graph V these figures show that only during the pregnant and early lactation periods do we find great differences between the sexes. It will be seen how at these times the graph of ♀H keeps more to the level of the males than the other two females. In the case of the males it will be seen that the entire absence or even restriction of the sex glands fails to have any effect on the calcium level. The graph of ♂E shows a sudden fall as in the case of females on the 28th November, but this is due to the vasectomy operation which was done on the 26th. Castration brought about the same fall, blood being taken a few hours after the operation. The graph shows the next value three days after the operation when the serum calcium was coming up to normal.

The results of the experiments on the younger 17 rabbits give data that are in line with those/

those recorded so far. The ages of these rabbits, progeny of ♀♀D - G, were at the commencement of bleeding:

Rabbits	I, II, III	6 - 7 weeks
"	IV -VI, XII - XIV	7 - 8 weeks
"	VII - XI	8 weeks
"	XV - XVII	12 -14 weeks.

Their data computed as before are:-

RABBIT	OBSERVATIONS	HIGHEST	LOWEST	TRUE MEAN with PROBABLE ERROR
♂ II	23	16.56	12.93	14.78 ± .154
♂ VII	28	15.48	11.45	13.79 ± .145
♂ X	27	16.86	11.98	14.59 ± .155
♂ XI	26	16.79	12.61	14.77 ± .157
♂ XII	26	17.08	12.39	14.54 ± .161
♂ XIII	26	16.5	12.5	14.89 ± .134
♂ XIV	26	16.97	12.99	15.08 ± .127
♂ XV	13	16.28	14.61	15.39 ± .091
♂ XVII	14	16.72	13.22	15.0 ± .225
♀ I	20	16.33	12.72	14.5 ± .139
♀ III	15	16.51	12.08	14.47 ± .195
♀ IV	19	15.74	12.6	14.26 ± .157
♀ V	19	16.17	12.7	14.26 ± .131
♀ VI	19	17.1	11.98	14.05 ± .17
♀ VIII	19	15.86	11.77	14.05 ± .17
♀ IX	18	14.64	11.41	13.06 ± .154
♀ XVI	18	16.53	11.33	14.61 ± .22
♂♂ II-XVII	209	17.72	11.45	14.69 ± .055
♀♀ I-XVI	147	17.1	11.33	14.12 ± .064

These/

These data are from the entire records of these animals, but again the periods of pregnancy and lactation have been omitted. On the average there appears to be sex-dimorphism. This is perhaps best seen if the difference with its probable error is computed.

$$\text{♂♂ II - XVII} \quad 14.69 \pm .055$$

$$\text{♀♀ I - XVI} \quad 14.12 \pm .064$$

$$\therefore \text{Difference} = .57 \pm \sqrt{.055^2 + .064^2}$$

$$= .57 \pm .084$$

$\therefore$ the difference is 6.78 times its probable error (.084).

The three groups of results then show that if the records are computed showing the values for each sex, the difference between the sexes is at least five times its probable error. If we take twice the error as the limit of significance (as suggested in FISHER'S book) then it is quite reasonable to suggest that there is sex-dimorphism in calcium-metabolism in rabbits.

The consideration of the limit of significance is arbitrary, but in the records submitted there/

there is indeed a slight difference between the sexes. RIDDLE AND HONEYWELL(1925)in their work on the pigeon found higher calcium-magnesium values in the female, but only on a basis of sex could they find a consistent grouping of values. The work of RIDDLE AND REINHART(1926)also on the pigeon, finds difference in the sexes at the ovulation periods. The results stated above, however, show that with the exception of the periods of pregnancy and lactation there is still a difference in the values for the sexes. These values show that in the males there is more serum-calcium than in the females.

Now, although the graphs show little or no difference in the sexes at normal times, during pregnancy and lactation the value for the females falls considerably below that of the males. This is perhaps natural for at these periods there is a considerable drain of calcium from the maternal organism. In conclusion it may be stated that contrary to the findings of RIDDLE AND HONEYWELL who found lower calcium values in the male than in the female pigeon and to RIDDLE AND REINHART who found greatly increased/

increased calcium values at ovulation periods in the pigeon, in the rabbit the calcium values are slightly higher in the male, always excepting the periods of pregnancy and lactation, but if these periods are included in the records the calcium values for the male are considerably above that of the female.

III./

III. COMPARISON in CALCIUM METABOLISM of DOE and LITTER.

The material employed for this experiment were the mother rabbits so far worked with and their litters. As far as the adult rabbits were concerned the required amount of blood for an estimation was quite conveniently obtained from the ear-veins, but in the case of the litters it was much more difficult. The litters could be bled from the ear when they were 4-6 weeks old. This would have meant a break in the series of records so it was decided to obtain the blood from the young litters by decapitation. This performed at intervals of days gave a series of results corresponding to the age of the litter.

Having carried out some decapitations and obtained results, it became evident that the series would be more complete if analyses of the blood of unborn foetuses were made. A worker in these laboratories, Dr. Tamura, carried out some unilateral laparotomies/

laparotomies for the writer on some of the pregnant rabbits. Three rabbits were at first operated on, each on the left side and the foetuses from the left horn of the uterus obtained, decapitated and the foetal blood collected. The result obtained from each such collection does not represent an individual result since each foetus would yield less than 1 c.c. blood. The result serves rather as an average for the whole litter obtained from that horn of the uterus. When the same rabbits were operated on the right side to obtain the remaining foetuses, it was found that these foetuses had died and were at the time of the second operation in a state of becoming absorbed. Accordingly only three results were obtained for unborn foetuses, corresponding to 23, 24 and 26 days of pregnancy. To complete this series a foetus from each litter was decapitated soon after birth and the serum-calcium determined.

PERIOD of GESTATION.	CALCIUM in m.gr. per 100 c.c. serum ♀	F_1	F_1 as percentage of ♀
23 days	14.52	12.08	83%
24 "	12.66	13.44	106%
26 "	8.44	14.7	174%
Soon after birth: i.e. at the end of days of gestation. (31 dys.	11.17	10.47	94%
(33 "	16.42	12.95	97%
(34 "	16.2	14.25	89%
(36 "	13.22	11.0	83%
Age of F_1			
3 days	12.17	11.12	91%
5 "	14.86	10.53	71%
6 "	15.56.	10.3	66%
7 "	13.81	11.58	84%
8 "	16.15	11.58	72%
9 "	14.74	11.23	76%
15 "	14.27	12.76	89%
16 "	14.74	11.23	76%
32 "	15.26	9.2	59%
34 "	13.3	13.89	104%
37 "	15.56	15.01	97%
40 "	14.35	13.57	95%
43 "	13.32	13.55	102%
46 "	14.47	14.58	101%
48 "	14.42	13.5	94%
49 "	13.76	13.37	97%
50 "	13.76	14.84	108%
52 "	15.13	14.36	95%
58 "	14.04	14.04	100%
65 "	13.36	14.31	107%
72 "	14.6	13.55	93%
79 "	14.79	13.42	91%
85 "	13.54	13.74	101%
91 "	13.9	14.26	103%
97 "	14.43	14.05	97%
103 "	14.94	15.27	102%
109 "	15.85	16.31	103%
115 "	13.65	14.0	103%
121 "	13.33	15.51	116%
127 "	14.76	14.76	100%

The entire results, given in a table, show that at the beginning of the fourth week of gestation maternal blood has very much more serum-calcium than the foetal, the foetal blood being 83% of the maternal. At 24 days of gestation the maternal blood has less serum-calcium than the foetal, while at 26days the foetal value is 174% that of the maternal.

Four rabbits were decapitated soon after birth and they show varying amounts of calcium which is, however, less than their respective mothers, the highest value being 3% less than the mother. The remaining figures in the table show the values for the litters from 3 days of age till they are 127 days old. Here it will be seen that calcium in the foetal blood is less than that of the mother until the age of 34 days is reached when the foetal serum-calcium is 4% higher than the maternal. The figures thereafter show that foetal blood has as much serum-calcium as the maternal. There are slight differences between foetal and maternal blood but these differences are comparable to those between one mother rabbit and another.

From/

From the figures it may be concluded that even in the gestation period foetal blood has less serum-calcium than maternal, until the last week of pregnancy when the foetal serum-calcium equals the maternal and surpasses it. This surpassing, however, is not due to a sudden increase in the foetal blood but rather to a sudden decrease in the maternal. It was shown previously that towards the close of prenancy there is a sudden drop in the maternal serum-calcium, this pregnancy minimum being reached a few days, or at the most 7 to 10 days before parturition, but after parturition there is as sudden a rise to normal. It will be noticed that at 26 days of gestation the foetal serum-calcium is 174% of the maternal. Now, in this case the figure for the mother 8.44 m.gm. was the minimum reached for the period of pregnancy, i.e. the serum-calcium had suddenly declined to the preparturition minimum. If the mother had not been operated on she would most probably have littered in 2,3, or 4 days. In spite of the operation, however, the rabbit littered, although a second operation had been performed 2 days after the first when it was seen that the foetuses in the/

the right uterine horn were dead. It is suggested that only at the close of pregnancy when the serum-calcium level of the mother is abnormal that the foetal serum-calcium is very mcuh higher than the maternal.

The table also shows that after parturition when the maternal value has become normal the maternal serum-calcium is higher than the foetal. This relation seems to persist till the foetus or young rabbit is about a month old when its serum-calcium level approaches that of its mother. Thereafter there is as much difference between foetal and maternal serum-calcium as between one full grown rabbit and another. The data are not numerous and therefore not as convincing as they might be but they lead one to conclude generally that even during gestation foetal serum-calcium is lower than the maternal. There is a steady increase with gestation but the value would still continue to be below that of the mother, but the maternal serum-calcium drops suddenly prior to parturition when the foetal serum-calcium appears to be very much in excess of the maternal.

When/

When the maternal value attains normality the foetal value is smaller but increases with age till at the age of about a month there is parity.

SUMMARY/

S U M M A R Y.

(1) Prior to pregnancy the calcium content in the blood of full-grown rabbits was found to vary between 13 and 15 m.gm. per 100 c.c. serum, with suggestions of a significantly regular rise and fall. During the early period of pregnancy no difference between the serum-calcium content of the pregnant and the non-pregnant does was found.

(2) 7 to 10 days before parturition the serum-calcium, then high, commences to fall and one, two or three days before parturition there is a further and more sudden fall to a minimum.

(3) After parturition the calcium content is restored to normal by a rise as sudden as was the previous fall, but about the 19th day of the lactation period, or thereabout, there is a second sudden fall to often a still lower minimum than was noted before parturition. Following this fall there is a sudden return to normal, but in some cases the return ends in a remarkable maximum of calcium in the serum, higher/

higher than any previously exhibited. Soon afterwards there is a return to normal, the calcium level then remaining steady till the end of the lactation period.

(4) Data collected from several rabbits of both sexes, excluding those relating to the periods of pregnancy and lactation, show little indication of any sex-dimorphism in calcium-metabolism. If, howeven, these data are computed and the true mean with probable error found for each sex it is seen that indeed there is a significant sex difference. The difference between the sexes was found to be more than five times its probable error, and if five times is taken as the limit of significance there is sex-dimorphism, the males having a higher serum-calcium content than the females.

(5) During the period of gestation foetal blood contains less serum-calcium than maternal until the last week of pregnancy when the foetal serum-calcium equals the maternal and in some cases surpasses it. This is to be explained by the sudden decrease in serum-calcium in the maternal blood.

After/

After parturition when the maternal value is again constant the maternal serum-calcium level is higher than the foetal. This relation persists till the young rabbit is about a month old when its serum-calcium level approaches that of its mother.

My grateful thanks are due to Dr. Crew for suggesting this line of research and for help and advice gladly given throughout its progress.

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