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THE SUSCEPTIBILITY OF VARIOUS GROUPS OF RATS TO EXPERIMENTAL HYPOCALCAEMIA

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Previous work, on cows, has shown that the period of late pregnancy is one of increased susceptibility to experimental hypocalcaemia. The experiments described in this paper were primarily designed to investigate the possibility that pregnant rats differed from non-pregnant rats in a similar way, and, if so, to investigate the fundamental basis of the phenomenon.

The main method used to investigate the difference between the various groups of rats was to measure their degree of susceptibility to an experimental hypocalcaemia induced by the intravenous infusion of a calcium chelating agent at a constant rate. The time for which the animals survived during the infusion was taken as a measure of their ability to withstand hypocalcaemia.

METHODS

The animals used in these experiments were either inbred hooded Norwegian or Wistar albino rats. All were adequately fed and in a good nutritional state, except in one experiment where the normal food was replaced for 24 hr before the experiment by a calcium-free diet consisting of pure glucose fed *ad lib.*, with distilled water replacing tap water for drinking.

Injection fluids. The disodium salt of ethylenediaminetetra-acetic acid (EDTA) was used as chelating agent in all the experiments described. In our first experiments a solution equivalent to 5·93 mg calcium per millilitre was used. This was prepared by dissolving 55·6 g of the disodium salt in about 500 ml. of distilled water, adjusting the pH to 6·8–7·0 with 5N-NaOH and making up to 1 l. with more distilled water. For later work this stock solution was diluted 1 in 2, 1 in 4 and 1 in 8 with normal saline.

Some experiments required calcium to be removed at a standard rate of 20 ng/sec/g (selected because it resulted in a survival time of convenient length). The injection was carried out as described below at the rate of 0·05 ml./20 sec and the adjustment for different rat weights was made by altering the concentration of EDTA in the injection fluid. A series of dilutions of EDTA were prepared for use in rats of various weights as shown in Table 1.

Injections. The rats were anaesthetized with ether and anaesthesia was continued throughout the injection. The tail vein was entered using a 30-gauge needle and 0·1–0·2 ml. of saline injected to ensure correct positioning. A syringe filled with EDTA was then carefully placed in the needle and the infusion started so as to deliver 0·05 ml./20 sec. After some time, depending on the rate of calcium removal, the breathing became heavy, then erratic and eventually ceased. When no further respirations occurred for 30 sec the animal was considered to have died; the time of the last breath was recorded as the end-point. Very few animals revived after this stage.

Symptoms of tetany due to hypocalcaemia were expected but these occurred only occasionally. When present they were represented by spasmodic muscle contractions, particularly towards the end of the injection.

Pregnant rats. Male and female rats were placed together overnight and vaginal smears examined in the morning for spermatozoa. Females successfully mated were put aside into a labelled cage to await experiment on either the twentieth or twenty-first day of pregnancy.

Parathyroidectomy. The rats were anaesthetized with open ether. Under strictly aseptic conditions, a mid-line incision was made in the neck and the muscles overlying the trachea separated. This revealed the two lobes of the thyroid gland with the parathyroids protruding at the surfaces. The latter were then removed, together with a little adjacent thyroid tissue, with fine-pointed scissors. The skin wound was closed with linen thread sutures.

TABLE 1. Dilutions of stock EDTA needed to remove 20 ng/sec/g from rats of various weights. Injection given at the rate of 0·15 ml./min

Weight of rat (g)	Dilution of EDTA stock solution (%)	Weight of rat (g)	Dilution of EDTA stock solution (%)
96–105	13·50	156–165	21·60
106–115	14·85	166–175	22·95
116–125	16·20	176–185	24·30
126–135	17·55	186–195	25·65
136–145	18·90	196–205	27·00
146–155	20·25		

Analyses. Plasma calcium was estimated volumetrically against standard disodium EDTA (0·75 g/l.), calcon being used as indicator (S.D. of replicate determinations is $\pm 1{\cdot}2\,\%$). Total calcium in tissues was estimated, after dry ashing and taking up in dilute hydrochloric acid, by the method of Clark & Collip (1925) (S.D. of replicate ashings and determinations is $\pm 3{\cdot}5\,\%$).

Determination of plasma volume and haematocrit. Blood volume determination was carried out by a dye-dilution technique with Evans Blue. Weighed quantities of Evans Blue solution were injected intravenously and the plasma volumes calculated by comparing with standard dilutions of dye. Haematocrits were determined by a standard technique in micro-haematocrit tubes.

The labelling of foetal calcium with calcium-47. Six pregnant rats were used in this experiment. Under open ether anaesthesia the abdominal cavity of each was opened and the gravid horns of the uterus withdrawn. Several of the foetuses were injected intraperitoneally with 0·05 ml. each of a solution containing 4 μc/ml. of ^{47}Ca in normal saline. The uterine horns were then replaced and the abdominal wound sutured with linen thread. Three of the pregnant rats treated in this way were then injected intravenously with 0·1 ml. of the stock EDTA solution/100 g to remove most of the plasma calcium. The remaining three rats served as controls.

Four hours later the rats were killed. Each foetus was preserved separately for direct radioactivity estimations. Estimation in maternal tissues was carried out after first dry-ashing a mince of tissue. Radioactivity was measured in a well-type γ-scintillation counter with a lead sleeve 5 mm thick to prevent the counting of the scandium-47 daughter of ^{47}Ca.

RESULTS

Survival time of rats during EDTA infusion at various rates

The survival times of rats during the intravenous injection of EDTA vary with the rate at which the EDTA is administered. This is presumably

the rate at which calcium is being removed, and is best expressed in ng/sec/g body weight. If the calcium removal rate is as high as 80 ng/sec/g of rat, the survival time is 120 sec. As the rate of calcium removal decreases the survival time increases, slowly at first and then more rapidly. If survival times at varying rates of calcium removal are plotted on arithmetical graph paper the resulting points lie in a curve (Fig. 1). However, if the data are plotted on log-log paper the points lie approximately on a straight line (Fig. 2). The coefficient of regression of this line is $-0{\cdot}971$ (S.E. $= \pm 0{\cdot}025$).

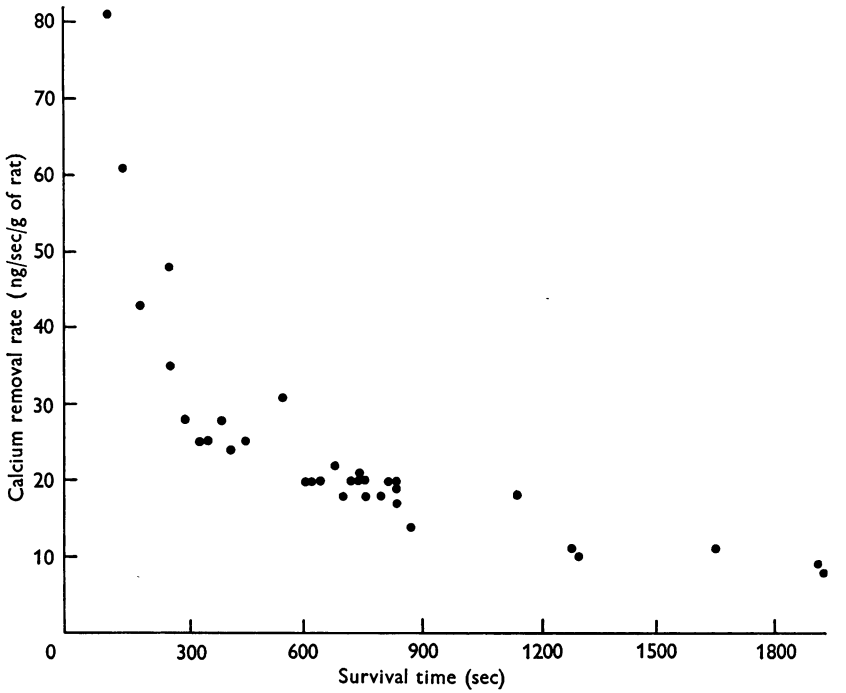

Fig. 1. The survival time of rats following calcium removal from the plasma at various rates.

Rats died when the plasma calcium concentration fell to 2 mg/100 ml. Presumably each rat has a pool of immediately available calcium which is progressively depleted during the injection until the fatal concentration is reached. If all the calcium available is in blood plasma then it is possible to calculate a theoretical series of survival times for various rates of calcium removal as follows. If a 200 g rat has 6 % of its body weight as blood plasma (see Table 4) and a plasma calcium concentration of 10 mg/100 ml. the total plasma calcium will be 1·2 mg. If death supervenes

when the concentration falls to 2 mg/100 ml. then 0·96 mg of plasma calcium is available for removal. Thus at a calcium removal rate of 100 ng/sec/g the rat should die in 960,000/(100 × 200) or 48 sec, at a rate of 50 ng/sec/g in 96 sec, at 25 ng/sec/g in 192 sec and so on. These theoretical survival times can be plotted in the same way as the experimentally obtained results, giving a curve on arithmetical graph paper and a line on log-log paper. The coefficient of regression of this line is −1·000, which is very close to the −0·971 obtained experimentally.

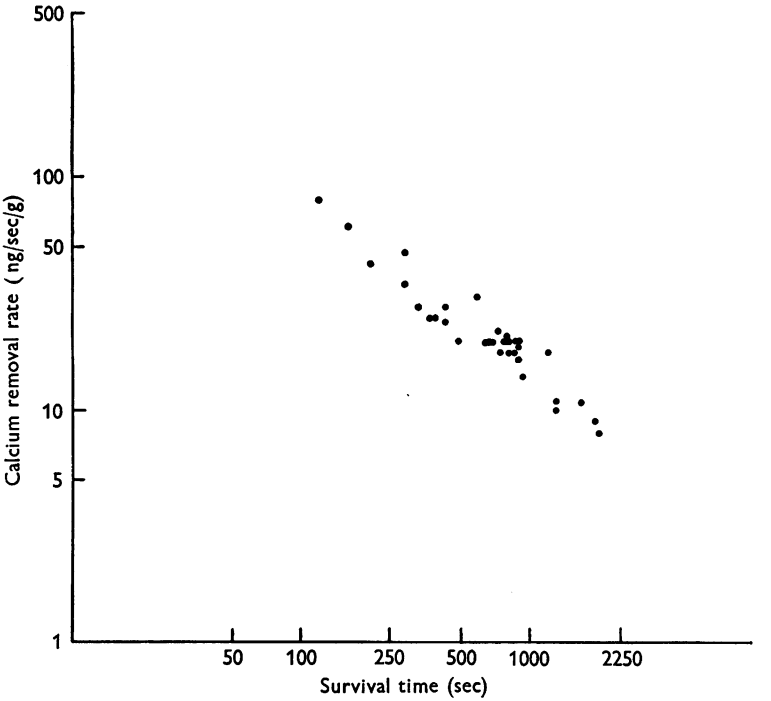

Fig. 2. The survival time of rats following calcium removal from the plasma at various rates as plotted on log-log paper.

Although the slope of the calculated line is a good fit to the experimental findings, all the survival times are much shorter. This implies that the pool of calcium available is greater than that present in the blood plasma alone. From Table 3 it can be seen that at a calcium removal rate of 20 ng/sec/g rats die in about 760 sec. Thus, in a 200 g rat a total of (20 × 760 × 200)/1,000,000 mg or 3·04 mg has been removed in this time. Assuming a concentration of 2 mg/100 ml. still remains in the reserve at death and that all the immediately available calcium reserve is in equilibrium with blood plasma at 10 mg/100 ml., then the total of immediately

available calcium is $(3{\cdot}04 \times 10)/8 = 3{\cdot}8$ mg or over 3 times the amount (1·2 mg) present in the blood plasma.

Survival times of normal rats and rats on calcium-free diet

When appropriate dilutions of the stock EDTA were used to remove calcium from the rats at the rate of 20 ng/sec/g, the survival times for normal rats were compared with those fed on a calcium-free diet (Table 2). The average survival times were similar but the variability of the results apparently increased. In view of this, normally fed animals were used in all later experiments.

TABLE 2. Average survival times of rats during experimental hypocalcaemia and after various treatments

Treatment or type of rat	No. of rats	Average survival time (sec ± S.E.)	Rate of calcium removal (ng/sec/g)
Normally fed hooded rat	8	762 ± 28	20
Calcium-free diet	8	750 ± 42	20
Parathyroid-hormone treated rats (hooded strain)	10	992 ± 21	20
Parathyroidectomized rats (hooded strain)	7	366 ± 49	20
Wistar albino rats over 170 g weight	9	840 ± 22	20
Wistar albino rats under 170 g weight	6	987 ± 50	20

The effect of strain of rat on the survival times

The figures in Table 2 suggest that there may be slight differences in survival times between the hooded and the Wistar Albino strains of rat. This is not significant statistically.

The effect of body weight on survival time

Rats of different weights were tested for their susceptibility to hypocalcaemia with EDTA solutions which removed 20 ng/sec/g. Rats of less than 170 g survived 987 sec, whereas animals over 170 g lived for an average of only 840 sec (Table 2). This difference is highly significant ($P < 0{\cdot}005$). Therefore in subsequent experiments rats of similar weight were chosen.

Effect of parathyroidectomy

With the 20 ng/sec/g doses of EDTA, parathyroidectomized rats survived only 366 sec. Compared with the first group of rats in Table 2 this effect is highly significant ($P < 0{\cdot}0001$).

Effect of parathyroid hormone treatment

Rats in this group (Table 2) were given 0·25 ml. (25 U.S.P. units) of Eli Lilly Parathormone 18 hr. before the test. The average survival time increased from 762 sec for controls to 992 sec for hormone-treated animals. This effect is highly significant ($P < 0·001$).

Effect of pregnancy

Our initial experiments involving pregnant rats were carried out with EDTA solution equivalent to Ca 1·48 mg/ml. (1 in 4 dilution of the stock EDTA solution) regardless of the body weight of the animal concerned. This was because all the animals, both pregnant and controls, came from the same batch and were of similar weight at mating. When the EDTA infusion was given the pregnant animals were heavier than the controls,

TABLE 3. Average survival times and weights of pregnant and non-pregnant rats during experimental hypocalcaemia

Status	No. of rats	Rate of calcium removal	Average survival time (sec ± S.E.)	Average weight of rat (g)	Average weight of uterine contents (g)
Pregnant	5	3·7 μg/sec	1280 ± 72	258	44
Non-pregnant	6	3·7 μg/sec	813 ± 39	197	—
Pregnant	6	20 ng/sec/g	750 ± 40	262	48
Non-pregnant	8	20 ng/sec/g	762 ± 27	208	—

because of the extra weight of the gravid uterine contents. Pregnant rats survived an average of 1280 sec, whereas non-pregnant rats lived for only 813 sec (Table 3). Much of this difference (highly significant, $P < 0·0001$) could be explained if it were assumed that the whole weight of the mother, including foetuses, was contributing to her survival.

Hence another experiment was carried out in which the EDTA solutions 20 ng/sec/g were used, thus taking into account the extra weight of the pregnant animals. It was then found that pregnant rats survived an average of 750 sec, very similar to the average survival time for non-pregnant rats (Table 3). These results might be explained in one or more of the following ways:

(1) There might be more calcium reserves in the plasma and body tissues of pregnant than non-pregnant rats.

(2) The calcium in foetal tissue might be available to the mother in time of need.

The following experiments were therefore carried out.

Estimation of plasma calcium in pregnant rats. As can be seen from Table 4, there is no difference between the plasma calcium concentration in pregnant and non-pregnant rats.

Plasma volume and blood haematocrit determination. Packed cell volume (haematocrit) is lower in pregnant rats than in similar non-pregnant animals (Table 4). This difference is highly significant ($P < 0.0001$). Moreover, plasma volume determination with Evans Blue showed a higher total plasma volume in pregnancy (highly significant, $P < 0.0001$). As none of the Evans Blue dye was present in foetal serum this must indicate an increase in the quantity of circulating plasma in the pregnant rat.

TABLE 4. Average weight, haematocrit plasma volume and tissue calcium values in pregnant and non-pregnant rats

Status	Total weight of rat (g ± S.E.)	Weight of foetus (g ± S.E.)	Packed red cell volume (% ± S.E.)	Total plasma volume (ml. ± S.E.)	Plasma volume as % body weight (% ± S.E.)
Pregnant	259 ± 5·8 (12 rats)	59·5 ± 3·7 (12 rats)	32·6 ± 0·58 (12 rats)	16·1 ± 0·29 (11 rats)	6·2 ± 0·48 (11 rats)
Non-pregnant	202 ± 6·3 (10 rats)	—	40·2 ± 1·45 (10 rats)	12·4 ± 0·54 (10 rats)	6·2 ± 0·44 (10 rats)

	Plasma calcium (mg/100 ml. ± S.E.)	Muscle calcium (mg/g)	Foetal calcium (mg/g)	Total calcium in the foetuses of each pregnant rat (mg)
Pregnant	9·23 ± 0·11 (10 rats)	0·135 (10 rats)	2·08 (7 rats)	82·9 (7 rats)
Non-pregnant	9·39 ± 0·12 (10 rats)	0·169 (7 rats)	—	—

Tissue calcium in pregnant rats. The calcium concentration is similar in muscles of pregnant animals to that in non-pregnant animals (Table 4). However, in pregnant animals there is an average of 82·9 mg of calcium in the foetal tissues.

The availability of foetal calcium to the mother. If calcium labelled with calcium-47 is injected into the foetus very little radioactivity penetrates across the placenta into the mother, even after sufficient EDTA has been given intravenously to remove most of the plasma calcium (Table 5). Severe hypocalcaemia was undoubtedly induced in these animals, because they showed evidence of respiratory distress before recovery. Even so, there was no difference between control and experimental groups in ^{47}Ca return from the foetus to the mother.

In summary, therefore, pregnancy appears to make no difference to susceptibility to hypocalcaemia in rats, even though up to one fifth of the

body weight is foetal tissue, from which calcium is not available. However, there is a greater reservoir of calcium in the mother's blood owing to an increase in the volume of circulating plasma.

DISCUSSION

There appear to be few descriptions of experimental hypocalcaemia in the literature. In one account, Stewart & Bowen (1951) used sodium oxalate to induce this condition in parathyroidectomized and normal dogs. The experimental dogs showed little tendency to recover after the injection, but good recoveries were obtained after the administration of parathyroid-gland extract. Similarly, Sanderson, Marshall & Wilson (1960) used EDTA to produce experimental hypocalcaemia in dogs. They found that normal dogs recovered rapidly after the injection but that parathyroidectomized animals did not do so. Campbell & Turner (1942) suggested that experimental hypocalcaemia in mice might be used for the assay of parathyroid hormone. They gave doses of 25% sodium citrate intraperitoneally and recorded the resulting incidence of convulsions which were mitigated by prior treatment with parathyroid hormone. Our own experiments on induced hypocalcaemia were carried out on cows (Payne, Sansom & Manston, 1963). Doses of sodium oxalate 20 or 24 mg/kg given intravenously at the rate of 1 g/min induced a safe but steep fall in the plasma calcium concentration. Cows in the last month of pregnancy were particularly susceptible to the treatment.

It was the primary purpose of the present experiments to study the effect of pregnancy on experimental hypocalcaemia in the laboratory rat. The disodium salt of ethylenediaminetetra-acetate was chosen as the chelating agent to remove calcium from the blood because some studies which will be reported in another paper (B. F. Sansom & J. M. Payne, unpublished) indicated that its effect on plasma calcium, unlike that of oxalate and citrate, was nearly instantaneous and complete. As our experiments on cattle had indicated that recovery curves after hypocalcaemia were difficult to interpret, a new type of experiment was planned which was not possible in cows. This involved the estimation of survival time during increasing hypocalcaemia induced by the steady infusion of EDTA intravenously. The survival time would depend on the rate at which calcium is removed by the EDTA and it was assumed that, at a given rate of calcium removal, survival times would be proportional to the success of the animal in mobilizing its calcium reserves. As the rate of calcium removal decreased the survival times increased and the quantitative relation was similar to that calculated on the basis that the rat's immediately available calcium reserves are a fixed quantity, which needs

to be exhausted to a minimal level before death supervenes. These reserves appear to be over three times the amount of calcium present in the blood plasma and probably include available calcium from tissues and tissue spaces. The presence or absence of mobilization of calcium from not immediately available sources such as intestinal contents and bones is unknown.

Contrary to expectations, the survival time was not influenced by feeding a calcium-free diet for 24 hr before the test. However, heavier rats tended to succumb quicker than lighter animals.

TABLE 5. Radioactivity of the mother after the injection of calcium-47 into the foetuses of rats with and without experimental hypocalcaemia

Rat group	Total dose to foetuses (counts/min)	Counts in mother's tissues (counts/min)	Per cent of total dose in mother
Control rats			
1	450,000	12,400	2·76
2	525,000	15,630	2·98
3	450,000	11,200	2·49
Rats after experimental hypocalcaemia			
4	375,000	10,570	2·82
5	375,000	11,100	2·96
6	300,000	10,220	3·42

Parathyroidectomy shortened the survival time considerably, as was expected from the published work mentioned above. Conversely, the administration of parathyroid hormone tended to increase the rats' survival time.

In the experiments involving pregnant rats it was found that, contrary to expectation, pregnancy did not decrease survival time, even though the foetal tissues accounted for about a fifth of the total body weight of the mothers. This seemed to imply that pregnant rats might be able to draw upon the calcium present in the foetus or that they had accumulated extra reserves within their own body tissues. The possibility that foetal calcium was returnable to the mother was disproved by the almost complete retention of ^{47}Ca injected into the foetus. Even after severe hypocalcaemic stress, no more of this isotope returned into the maternal tissues than in control animals. Analysis of the calcium concentration in the mother's muscle and plasma indicated that this was not higher than normal during pregnancy. However, haematocrit and plasma volume determinations indicated that there was an increased quantity of plasma circulating in the mother's tissues comparable on a percentage body-weight basis to the extra amount of foetal tissue present in the uterus. This means that there is an effective increase in the total amount of circulating plasma

calcium in the pregnant rat. We suggest that an increased plasma volume might be a factor in helping the pregnant rat to cope with the nutritional stress caused by the rapidly growing foetuses during the last days of pregnancy.

SUMMARY

1. The survival times of rats undergoing experimental hypocalcaemia induced by the steady intravenous injection of EDTA have been studied.

2. Varying the rate of EDTA infusion influences the time of death in a manner that would be expected from theoretical calculations. A calcium-free diet did not influence the survival time. Heavier animals tended to die more quickly than lighter ones. The prior injection of parathyroid hormone tended to increase the survival time whilst parathyroidectomy decreased it considerably.

3. Pregnancy did not affect the survival time even though one fifth of the pregnant rat's weight is foetal tissue. Injections of ^{47}Ca into the foetus showed that foetal calcium was not returnable to the mother even under the stress of severe hypocalcaemia. No additional concentrations of calcium were detected in the mothers' muscle or blood but plasma volume determinations showed that there was a greater quantity of circulating plasma and thus of immediately available calcium in the pregnant rat.

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