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# Calcium and phosphorus metabolism in the grey–lethal mouse

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## 1. INTRODUCTION

The grey–lethal mutation in the mouse (symbol *gl*; L.G. IV; Grüneberg, 1935, 1936, 1937, 1938; Bateman, 1954) affects the skeleton in its entirety. In the grey–lethal (*gl*/*gl*) there is a complete absence of secondary bone absorption with persistence of spongiosa spicules, characteristic shape anomalies of individual bones, poor development of periosteal bone and incomplete calcification of the skeleton. Failure of bone absorption in the dental crypts leads to non-eruption of the teeth and death of the animals soon after weaning. The grey colour of the coat is now known to be due to clumping rather than absence of the yellow pigment or phaeomelanin (Grüneberg, 1966). The first biochemical studies on the grey–lethal mouse were made by Watchorn (1938) who discovered that these animals have a low serum phosphorus. The present paper is part of a detailed metabolic study undertaken to elucidate the physiological defect underlying the systemic morphological anomalies of the grey-lethal mouse.

## 2. METHODS

*Animals.* Normal and grey–lethal mice came from matings between heterozygotes (+/*gl*) from a stock which is not highly inbred. All mice were fed on diet No. 86 of the Rowett Research Institute, Aberdeen, as supplied by the North-Eastern Agricultural Co-operative Society Ltd., Aberdeen. In experiments, grey–lethal mice were paired with normals from the same litter and the same sex whenever possible, although no sex differences were noted in the variables measured. Mice in the age range 10–16 days which were being nursed by their mothers were used for most experiments in order to ensure optimal nutrition.

*Tissues.* Animals were sacrificed by decapitation, carotidojugular blood collected and the cells spun down for serum analysis. Calcium in serum was determined according to Spandrio (1965) and phosphorus according to Chen, Toribara & Warner (1956). Serum acid and alkaline phosphatases were determined initially by the method of Bessey, Lowry & Brock (1946) and latterly according to Kind & King (1954). Bones were prepared for analysis by dissecting out both femurs from each mouse and carefully removing all adhering tissue. The cleaned bones were extracted in an ether–ethanol mixture (1:1) for 24 h followed by an ether extraction for a further 24 h. The defatted bones were then dried in an oven at 110 °C for

several hours and weighed. Ashing was performed in a muffle furnace at 500 °C for about 24 h. The weighed samples were dissolved quantitatively in 100 ml. of 0·01 N-HCl. The ash solution was analysed for Ca and for P after conversion of the latter to orthophosphate by heating aliquots with 1 N-HCl at 100 °C for 10 min.

*In vitro studies. In vitro* steady-state balances of Ca and P were studied in living and heat-inactivated bone from normal and grey–lethal animals. The femurs and calvaria were dissected out and cleaned of all other tissue. They were washed in iced medium, quickly blotted dry with filter paper and weighed. The weighed samples were then placed in incubating flasks containing iced medium and stored in ice until the start of the experiment, usually about 10–15 min later. About 100 mg of tissue was used in each flask, representing the pooled calvaria or femurs from three mice. All incubations were carried out in 2·5 ml. of Ca-free Krebs–Ringer bicarbonate medium, buffered to pH 7·4. The phosphate content of this medium was usually about 0·4 mM. Glucose was added to a final concentration of 11·1 mM. Penicillin and streptomycin to a final concentration of 5 units/ml. and 10 units/ml. respectively were added to prevent bacterial growth. All media were aerated with the appropriate gas before being placed in the flasks. All experiments were carried out in stoppered flasks in an atmosphere of 95 % $O_2$ and 5 % $CO_2$ in a metabolic shaker at 37 °C and shaking at 110 strokes/min. In experiments in which the bone was inactivated the procedure was as follows: 2·5 ml. of glucose-free medium was brought to the boil, removed from the flame and the bone samples were submerged in the hot fluid for 3 min. Failure of these samples to utilize glucose and produce lactate was taken as evidence of inactivation. Lactate was determined according to Barker & Summerson (1941). In the *in vitro* experiments Ca was determined on an Oxford Titrator, using a modification of the method of Bett & Frazer (1959). DNA and RNA were measured on the hot TCA extract of decalcified bone by the method of Schneider (1957). Decalcification was carried out by extraction for three successive 24 h periods in 10 ml. of 10 % EDTA adjusted to pH 7·5, containing 0·1 % chloroform to prevent bacterial contamination. The extraction was done at 0–5 °C with constant agitation.

## 3. RESULTS

These experiments were designed to discover any anomalies that might exist between the Ca and P metabolism of the grey–lethal mutant and that of its normal litter-mate control. Table 1 summarizes the values of the serum Ca, P and total blood P in normal and grey–lethal animals. The mutant animal is hypocalcaemic and there is a marked hypophosphataemia. The grey–lethal mouse showed a consistently low mean inorganic P when compared with controls, with a marked depression in many individuals. The mean serum P value of 6·16 mg % is 43·8 % ($P < 0·001$) lower than that of the normal mouse. The consistency of this hypocalcaemic hypophosphataemic effect is shown in Fig. 1, where the mean Ca:P ratio for normal and grey–lethal animals is plotted over a 10-day period from 10 to 20 days of age.

Table 2 compares the values of the serum acid and alkaline phosphatases. The acid phosphatase values conform to the normal but there is a very significant elevation (+98%, $P < 0{\cdot}001$) in the alkaline phosphatase of the mutant mouse. Alkaline phosphatase was also determined in mice that had been starved for 24 h to eliminate the factor involving fat absorption (Gould, 1944), which is known to effect serum phosphatase levels. The values obtained, however, were essentially

Table 1. *Serum Ca, P and total blood P in normal and mutant mice 10–23 days old*

| | Means | | Difference: normal − mutant | |
|---|---|---|---|---|
| | Normal | Mutant | ± S.E. | *P* |
| Ca (mg %) | 9·40 (17) | 8·70 (17) | 0·70 ± 0·25 | < 0·02 |
| P (mg %) | 10·98 (19) | 6·16 (19) | 4·82 ± 0·41 | < 0·001 |
| Total blood P (mg %) | 55·54 (8) | 33·59 (8) | 21·95 ± 3·64 | < 0·001 |

In this and subsequent tables the significance of the difference between the means for normal and grey–lethal mice was judged by Student's *t* test for paired comparisons between litter-mates. *P* values are given; where *P* is greater than 0·05 the values are quoted as not significant (NS). Parentheses enclose the numbers of pairs in each group.

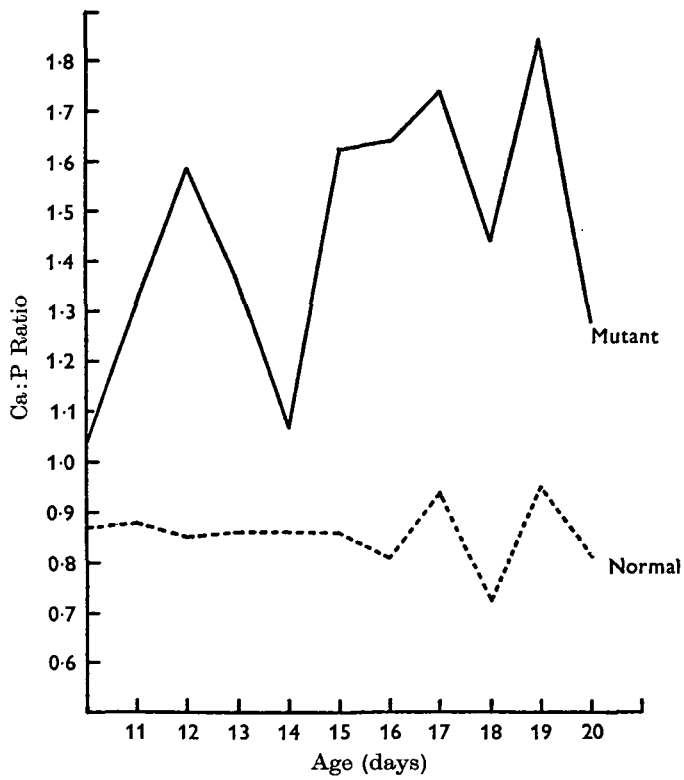


Fig. 1. Mean serum Ca:P ratio in normal and mutant mice ranging in age from 10 to 20 days.

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the same as in fed mice and a difference of the same magnitude was found between the normal and mutant levels of enzyme.

A detailed bone analysis was carried out on femurs from normal and grey–lethal animals aged 10–23 days. Grey–lethal bone appears much more dense than that of its normal litter-mate and this is confirmed in the ash content as shown in Table 3. When expressed as a percentage of the dry weight of the femurs, mutant ash content is 19·5% ($P < 0·02$) higher than the normal. This increased ash weight

Table 2. *Serum acid and alkaline phosphatase in normal and mutant mice 10–23 days old*

| | Means | | Difference: normal − mutant ± S.E. | $P$ |
|---|---|---|---|---|
| | Normal | Mutant | | |
| Acid phosphatase | 8·63 (14) | 9·10 (14) | −0·4 ± 1·10 | NS |
| Alkaline phosphatase | 35·19 (9) | 69·69 (9) | −34·50 ± 3·64 | < 0·001 |

Results are expressed in King Armstrong units/100 ml. A K.A. 'unit' is defined as the amount of enzyme which will liberate 1 mg of phenol from phenyl phosphate in a given time under the conditions of the test.

Table 3. *Bone analysis on femurs from normal and mutant mice*

| | Means | | Difference: normal − mutant + S.E. | $P$ |
|---|---|---|---|---|
| | Normal | Mutant | | |
| Bone ash content (% dry weight of femurs) | 39·71 (13) | 47·08 (13) | −7·37 ± 2·43 | < 0·02 |
| Ca (% ash) | 32·94 (13) | 38·60 (13) | −5·66 ± 2·03 | < 0·02 |
| P (% ash) | 19·48 (13) | 19·01 (13) | 0·47 ± 0·83 | NS |

Table 4. *Concentrations of calcium and phosphate in incubation medium maintained at steady state by living and heat-inactivated femur and calvarium from normal and grey–lethal animals*

| | Means | | Difference: normal − mutant ± S.E. | $P$ |
|---|---|---|---|---|
| | Normal | Mutant | | |
| | Living bone | | | |
| Calvarium: | | | | |
| Phosphate (mM/l.) | 1·37 (14) | 1·35 (14) | 0·02 ± 0·04 | NS |
| Calcium (mM/l.) | 1·09 (6) | 1·05 (6) | 0·04 ± 0·05 | NS |
| Femur | | | | |
| Phosphate (mM/l.) | 2·08 (7) | 1·82 (7) | 0·26 ± 0·16 | NS |
| Calcium (mM/l.) | 1·73 (5) | 1·64 (5) | 0·09 ± 0·14 | NS |
| | Metabolically dead bone | | | |
| Calvarium | | | | |
| Phosphate (mM/l.) | 1·06 (14) | 1·10 (14) | −0·04 ± 0·04 | NS |
| Calcium (mM/l.) | 0·39 (6) | 0·50 (6) | −0·11 ± 0·03 | < 0·05 |
| Femur | | | | |
| Phosphate (mM/l.) | 1·59 (7) | 1·61 (7) | −0·02 ± 0·10 | NS |
| Calcium (mM/l.) | 0·78 (5) | 1·00 (5) | −0·22 ± 0·05 | < 0·02 |

is probably accounted for by the elevated Ca content found in the ash of the grey–lethal bone (+17·1 %, $P < 0{\cdot}02$). The P content of the ash was found to be the same in both.

The role played by the bone in maintaining Ca and P levels in the serum was examined by measuring *in vitro* the Ca and P concentrations reached under steady-state conditions in glucose containing Krebs–Ringer medium in which calvarium or femur was incubated for prolonged periods. In this system the concentration of both ions reached a plateau value after about 6 h, suggesting that a steady-state distribution between bone sample and its surrounding medium had been achieved. Incubation was continued for a further 2 h and the results listed in Table 4 give the values after 8 h of incubation. In this system the concentration of these ions maintained by heat-inactivated samples of bone can be considered to be representative of the solubility of the bone-mineral phase, while any increment in concentration above this value maintained by living samples can be considered to be due to active cellular metabolism in the bone (Schartum & Nichols, 1961; Vaes & Nichols, 1961).

Table 5. *DNA and RNA content of fresh bone from normal and grey–lethal mice*

| | Means | | Difference: normal − mutant ± S.E. | $P$ |
|---|---|---|---|---|
| | Normal | Mutant | | |
| DNA (μg/100 mg) | 445·2 (12) | 517·7 (12) | −72·5 ± 62·2 | NS |
| RNA (μg/100 mg) | 116·3 (11) | 197·4 (11) | −81·1 ± 31·5 | < 0·05 |
| RNA/DNA | 0·27 (11) | 0·37 (11) | −0·10 ± 0·07 | NS |

From the data presented in Table 4 it can be seen that normal and grey–lethal bone maintain the same steady-state distribution of Ca and P in their external media. However in the heat-inactivated mutant bone an interesting situation arises. The Ca in the medium due to passive ion solubility is increased 28 % above the normal control both in the case of femur ($P < 0{\cdot}02$) and calvarium ($P < 0{\cdot}05$). Therefore, although the final steady-state values of Ca achieved do not differ significantly, that part of the concentration which is due to active cellular metabolism is considerably greater in normal than in mutant bone. Thus, the mineral of bones from mutant animals appears to have increased solubility independent of the metabolism of surviving cells.

A comparison of results of metabolic studies such as detailed above, using the tissue weight as reference, is valid only if the cell concentration in the tissues is constant and comparable. DNA content was therefore used as an index of bone-cell concentration. DNA content per unit wet weight of tissue (Table 5) is slightly, although not significantly higher in mutant bone. RNA was also measured at the same time and mutant bone was found to contain somewhat greater quantities. However, the RNA/DNA ratio in the two types of bone does not differ significantly.

## 4. DISCUSSION

The experiments outlined above not only confirm certain aspects of this mutation previously discussed by others (Grüneberg, 1935; Watchorn, 1938), but have brought to light some new interesting features of this hereditary osteopetrosis in the mouse. The lack of bone resorption concomitant with the hypocalcaemia would point to a hypoparathyroidism but the hypophosphataemia militates against this and would suggest rather a hyperparathyroidism. This is also suggested by the finding of a high serum alkaline phosphatase. It has been noted that administration of parathormone increases the level of this enzyme in dogs (Cantarow, Brundage & Housel, 1937), in guinea-pigs (Ambroso, Zinicola & Lastella, 1958) and in normal and parathyroidectomized rats (Cenciotti, Mariotti & Zoli, 1959; Mouzas & Weiss, 1961).

One of the well-established direct effects of parathormone is its ability to bring about the resorption of bone *in vivo* and *in vitro* (Barnicot, 1948; Chang, 1951; Gaillard, 1961; Raisz, 1963). Taking this point as criterion of the action of this hormone it would appear that in the grey–lethal the hormone is (*a*) present in reduced amounts, (*b*) inactivated in some way, or (*c*) inhibited in its action by another factor. The latter two points are the most reasonable in view of the fact that grey–lethal mice tolerate a dosage level of parathormone which is lethal to normal mice (Barnicot, 1945; Walker, 1966*a*).

In 1963 Hirsch, Gauthier & Munson reported the discovery of thyrocalcitonin, a polypeptide hormone of the thyroid glands. This has subsequently been confirmed by Foster, Baghdiantz, Kumar, Slack, Soliman & MacIntyre (1964). Two essential features of this hormone are its ability to lower serum Ca and inhibit bone absorption (Friedman & Raisz, 1965; Aliapoulios, Goldhaber & Munson, 1966; Foster, Doyle, Bordier & Matrajt, 1966; Wase, Peterson, Rickes & Soliwski, 1966). Since both these effects are found in grey–lethal mice it is tempting to postulate an involvement of this hormone in the aetiology of this osteopetrosis. This possibility is strengthened by evidence of increased parathormone activity. Gaillard (1966) and Hirsch (1967) have shown that the effects of thyrocalcitonin are more pronounced when resorption is stimulated by parathormone. Since thyrocalcitonin has been shown to act directly on bone an increased production of this hormone would account for our findings of an absence of bone resorption, a lowered serum Ca and P and a decreased active cellular metabolism. In an attempt to counterbalance this effect, an increased production of parathormone would accentuate the bone and serum effects and would account for the raised serum alkaline phosphatase and the increased passive ion solubility found in the experiments *in vitro*. Schartum & Nichols (1961) showed that treatment with parathormone effected such an increase in the passive solubility of bone mineral. An increased parathormone secretion would also explain the altered citrate metabolism found in this mutant system (Murphy, 1968).

While the studies to date on the Ca and P metabolism in the grey–lethal mouse do not reveal the exact nature of the physiological defect responsible for the

generalized lack of bone resorption they do, however, put forward some interesting possibilities. These results indicate the situation we would find if thyrocalcitonin blocked resorption at a site which is basic to the entire resorption mechanism. Walker (1966*b*) found a high concentration of parafollicular light cells in the thyroid gland of parathormone stimulated grey–lethal mice. These are the cells which Foster, MacIntyre & Pearse (1964) postulate to be the source of calcitonin. This fact taken in conjunction with our results strengthens the hypothesis that osteopetrosis in this mutant is due to inhibition of bone resorption by thyrocalcitonin.

SUMMARY

Ca and P metabolism in the grey–lethal mutant mouse has been investigated. This animal is hypocalcaemic and hypophosphataemic and the serum alkaline phosphatase is elevated far above the normal. Increased bone ash content has been attributed to an elevated bone Ca. From *in vitro* experiments involving steady-state distributions of calcium and phosphate it is concluded that active bone metabolism is reduced in mutant bone. This reduced ratio may be attributed to an increased passive ion solubility.

These results tend to the assumption of an hormonal imbalance in this mutant system. An increased production of thyrocalcitonin has been postulated to account for these findings.

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