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Zinc and Magnesium in the Uterus of the Pregnant and Pseudopregnant Mouse and the Effects of Mg^{2+} Ions on Uterine Alkaline Phosphatase

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Abstract

The levels of zinc and magnesium in the mouse uterus during early pregnancy and pseudopregnancy were determined using atomic absorption spectroscopy techniques. The total zinc and magnesium content of the uterus increased between days 5 and 12 of pregnancy and between days 5 and 9 of pseudopregnancy when decidual cells were present. However, the metals were not accumulated at a rate sufficient to match increases in uterine weight and constant concentrations (micrograms of metals per gram wet weight of tissue) were not maintained over the various reproductive stages studied. The accumulation of the metals was associated with the presence of decidual cells, and non-decidualized horns of pseudopregnant mice failed to increase their total content of zinc and magnesium between days 5 and 9. The magnesium content of each uterus was usually between 5- and 13-fold greater than the total zinc content.

Mg^{2+} in low concentrations (0 – 2 mM) stimulated both the pyrophosphatase and orthophosphatase activities of purified preparations of the mouse uterine metalloenzyme, alkaline phosphatase. Higher concentrations (up to 8 mM) of the cation decreased pyrophosphatase activity but did not alter orthophosphatase activity. Mg^{2+} was more effective, however, in increasing the orthophosphatase activity of the enzyme and its stimulating effects in this case were greater in carbonate–bicarbonate buffer than in glycine–NaOH buffer. Mg^{2+} did not significantly influence apparent K_m values or the response of the enzyme to changes in temperature. Zn^{2+}, however, was required to maintain the stability of alkaline phosphatase apoenzyme preparations.

It was concluded that during normal pregnancy and pseudopregnancy zinc and magnesium would always be present in amounts considerably greater than those required to saturate alkaline phosphatase for full catalytic activity. Thus, while the metals exert major effects on the activity and stability of the enzyme *in vitro*, they may not be major factors involved in the *in utero* regulation of the enzyme during early pregnancy.

Introduction

Zinc and magnesium are essential metals in mammalian reproduction and are required for the successful implantation of the conceptus, the normal progression of pregnancy (Cox *et al.* 1969; Caldwell *et al.* 1973), and the modulation of the uterine response to oxytocins (Fraser 1939; Krejči and Poláček 1968). The levels of these metals in the uterus of a number of species are influenced by ovarian hormones and change with variations in reproductive status (Walaas 1950; Emanuel and Oakey 1969; Aitken 1974). Lutwak-Mann and McIntosh (1969) suggest that, during early pregnancy in the rabbit, zinc is probably shifted from body deposits to the uterus to meet requirements of the endometrial-mediated phase of foetal development.

A number of metalloenzymes that need either zinc or zinc and magnesium for catalytic activity display marked changes in activity in the mammalian uterus during early pregnancy and possibly play important roles in early embryonic development (see Lutwak-Mann and McIntosh 1969; Murdoch *et al.* 1978). One such enzyme is alkaline phosphatase (orthophosphoric monoester phosphohydrolase, EC 3.1.3.1) which in the mouse uterus is associated with the induction of the decidual cell reaction (Finn and Hinchliffe 1964) and requires both Zn^{2+} and Mg^{2+} ions for hydrolytic activity (Murdoch *et al.* 1980). The mechanisms responsible for the regulation of this enzyme have not been resolved, but it is possible that alterations in the availability of Zn^{2+} and Mg^{2+} ions *in utero* may be involved. Thus, it might be expected from the studies of Iqbal (1970) that, in the presence of low levels of cations, inactive apoenzyme forms of alkaline phosphatase may be synthesized which can later be activated by exposure to adequate levels of the metals.

In order to assess whether Zn^{2+} and Mg^{2+} ions are potential regulators of the enzyme *in utero*, the levels of these metals in mouse uterine tissue were determined at various stages of pregnancy and pseudopregnancy when alkaline phosphatase displays major changes in activity (see Murdoch *et al.* 1978). Because Mg^{2+} ions in particular are capable of stimulating the activity of various purified alkaline phosphatases from a number of sources (Brunel and Cathala 1973; Gerbitz 1977), the effects of this cation on mouse uterine alkaline phosphatase were also examined in the present investigation. Two different buffer systems were employed in the study of Mg^{2+} effects since it is recognized that reaction conditions can sometimes influence the catalytic properties of alkaline phosphatases and their response to cations (Rej 1977).

Materials and Methods

Animals

Female Quackenbush strain mice, aged 8-12 weeks, were used in all experiments and were housed as previously described (Murdoch *et al.* 1978). Pregnancy or pseudopregnancy were brought about by pairing the females with intact or vasectomized males, respectively. The females were examined for copulation plugs each morning and the day of finding a plug was designated as day 1 or the first day of pregnancy or pseudopregnancy. Deciduomata were induced in the left uterine horns of pseudopregnant mice by the intraluminal injection of 30 μl of peanut oil, as described by Finn and Hinchliffe (1964), after anaesthesia with pentobarbitone sodium (Nembutal). The oil injection was administered at the uterotubal junction between 1500 and 1630 h on day 4 of pseudopregnancy. The mice were killed by cervical dislocation at 0900 h on days 5–9 of pseudopregnancy and on days 2–12 of pregnancy.

Preparation of Uterine Tissue for Atomic Absorption Spectroscopy

At autopsy the uteri were removed and dissected free of fatty and connective tissue and of the attached oviducts and cervix. The embryonic tissue at each implantation site along the uterus was carefully removed by dissection. Care was taken to leave the placenta intact to avoid the possibility of removing decidualized stromal tissue. Each uterus was minced with fine scissors, weighed and then digested in 1·0 ml of fuming nitric acid for 2 h at about 55°C. Following digestion, one drop of H_2O_2 was added and the mixture was evaporated to a volume of about 0·3 ml under a stream of air. The concentrated preparation was then reconstituted in 4 ml of 0·01 M HCl and divided into two equal parts. One part was used for the determination of zinc while the other was diluted in five parts of an aqueous solution containing 550 μg/ml lanthanum just prior to subjecting the samples to the determination of magnesium. This concentration of lanthanum is less than the 0·1% recommended by the U.S. National Bureau of Standards (Rains 1977).

Uteri from pseudopregnant mice were also prepared by this method with the exception that the right and left horns were individually excised at the time of autopsy and processed separately. Whole uterine preparations were used to assay zinc and magnesium levels in all cases because initial attempts to separate myometrial and endometrial tissues caused the loss of variable amounts of extracellular fluid which could act as an important source of the cations for the uterine cells.

The levels of zinc and magnesium in the preparations were determined using a Pye Unicam SP-1900 atomic absorption spectrometer.

Enzyme, Apoenzyme and Zn-apoenzyme Preparations

Purified preparations of alkaline phosphatase and apoenzyme preparations, containing no residual activity in the absence of added cations, were obtained from the uteri of mice on day 7 of pregnancy as described in a previous paper (Murdoch *et al.* 1980). Apoenzyme preparations containing zinc (Zn-apoenzyme) but having no residual activity in the absence of added Mg^{2+} ions were obtained by incubating the apoenzyme (10 μg of protein) in a volume of 0·1 ml of 32 mM Tris–HCl buffer, pH 8·0, containing 1·0 mM $ZnCl_2$ for 1 h at 25°C. Following incubation the preparations were dialysed against two changes of Zn-free buffer solution for 24 h at 4°C. Activation of the apoenzyme and Zn-apoenzyme preparations was achieved by incubation for 10 min at 25°C in buffer solutions containing either 0·25 mM $ZnCl_2$ and 2·0 mM $MgCl_2$ or 2·0 mM $MgCl_2$, respectively.

Enzyme Assays

Alkaline phosphatase activity was assayed either by the spectrophotometric measurement of hydrolysis of *p*-nitrophenyl phosphate at 405 nm or by measuring the amount of orthophosphate (Pi) liberated from various substrates in the presence of the enzyme (Murdoch *et al.* 1980). The buffer systems employed for assay were 50 mM glycine–NaOH, pH 10·5, and 50 mM carbonate–bicarbonate, pH 10·5. Units of enzyme activity are defined either as micromoles of substrate hydrolysed per minute or as micromoles of Pi liberated per minute.

In all experiments before alkaline phosphatase activity was assayed, the enzyme was incubated for 10 min at 25°C in a total volume of 0·1 ml of 32 mM Tris–HCl buffer, pH 8·0, containing $MgCl_2$ at concentrations of 0, 0·02, 0·2 and 2·0 mM, unless stated otherwise. Enzyme activity was then assayed as described above with care being taken to ensure that the final $MgCl_2$ concentration was not altered by dilution when the substrate–buffer reaction mixture was added.

K_m (Michaelis constant) values and the response of the enzyme to changes in temperature were determined as described in a previous paper (Murdoch *et al.* 1980).

Replication of Experiments and Statistical Methods

All zinc and magnesium determinations were carried out in duplicate and were replicated six times with different preparations on each occasion. The raw data were converted to logarithms and submitted to standard analysis of variance. When applicable, all main effects and their first-order interactions were isolated and tested for significance using the within-group error mean square to calculate variance ratios. The multiple-range test (Duncan 1955) was used to compare levels of cations at the various reproductive stages studied.

Studies of the effects of Mg^{2+} on alkaline phosphatase preparations were also carried out in duplicate and were replicated three times with different preparations on each occasion. The significance of results was again assessed by analysis of variance or by employing Student's *t*-test.

All values given in the text and figures are means ± standard error of the mean.

Results

Levels of Zinc and Magnesium in the Pregnant Uterus

Figs 1*a* and 1*b* show the concentration (in micrograms per gram wet weight of tissue) of zinc and magnesium in the mouse uterus between days 2 and 12 of pregnancy. Although the concentration of magnesium was usually about 10-fold greater than that of zinc, the levels of both metals followed a similar pattern as pregnancy progressed. Thus, maximum values were recorded in the early stages of pregnancy and thereafter decreased to reach minimum levels on day 12.

When these data were expressed as total micrograms of metal per uterus (Figs 1*c* and 1*d*), the magnesium content of each uterus was again between 5- and 13-fold greater than the total zinc content, but the patterns of changing levels with advancing pregnancy differed from those described above. In this case, the total zinc content of the uterus was low on days 2 and 3 of pregnancy but increased significantly

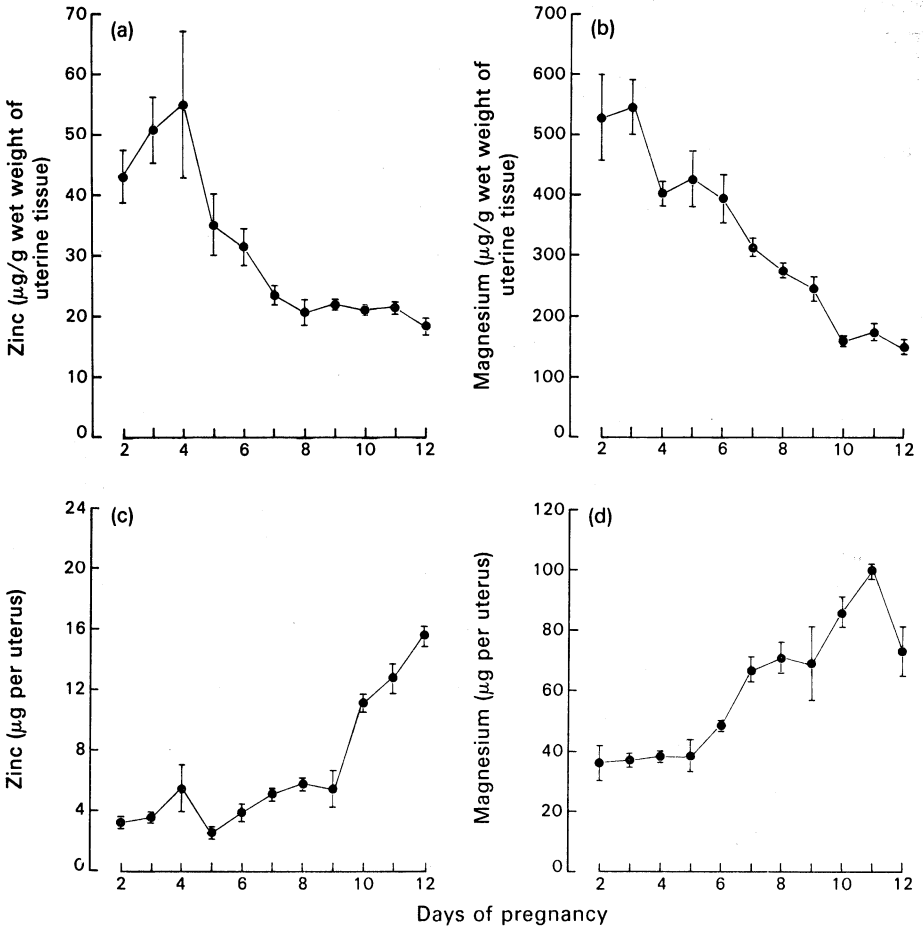


Fig. 1. Concentrations of zinc (*a*) and magnesium (*b*) and total zinc (*c*) and magnesium (*d*) content in the uterus of the mouse during early pregnancy. The multiple-range test gave the following ranking (at the 5% level) on the various days of pregnancy for the metal concentrations and content:

Zinc concn: 4 = 3 = 2; 4 > 5 = 6 > 7 = 8 = 9 = 10 = 11 > 12
Magnesium concn: 2 = 3 > 4 = 5 = 6 > 7 > 8 > 9 > 10 = 11 = 12
Zinc content: 12 > 11 > 10 > 9 = 8 = 7 = 6 = 4 > 5; 4 > 3 = 2
Magnesium content: 11 > 10 > 12 = 9 = 8 = 7 > 6 > 5 = 4 = 3 = 2

($P < 0{\cdot}05$) on day 4 to fall once again on day 5. After day 5 the total zinc content gradually increased to reach maximum levels on day 12. The total magnesium content of the uterus followed a similar profile, but no significant changes were detected between days 2 and 5. The maximum magnesium content was detected on day 11 of pregnancy and not on day 12 as was observed with zinc.

Levels of Zinc and Magnesium in the Pseudopregnant Uterus

The concentrations (in micrograms per gram wet weight of tissue) of both zinc and magnesium were significantly higher ($P < 0{\cdot}01$) in the non-decidualized (right)

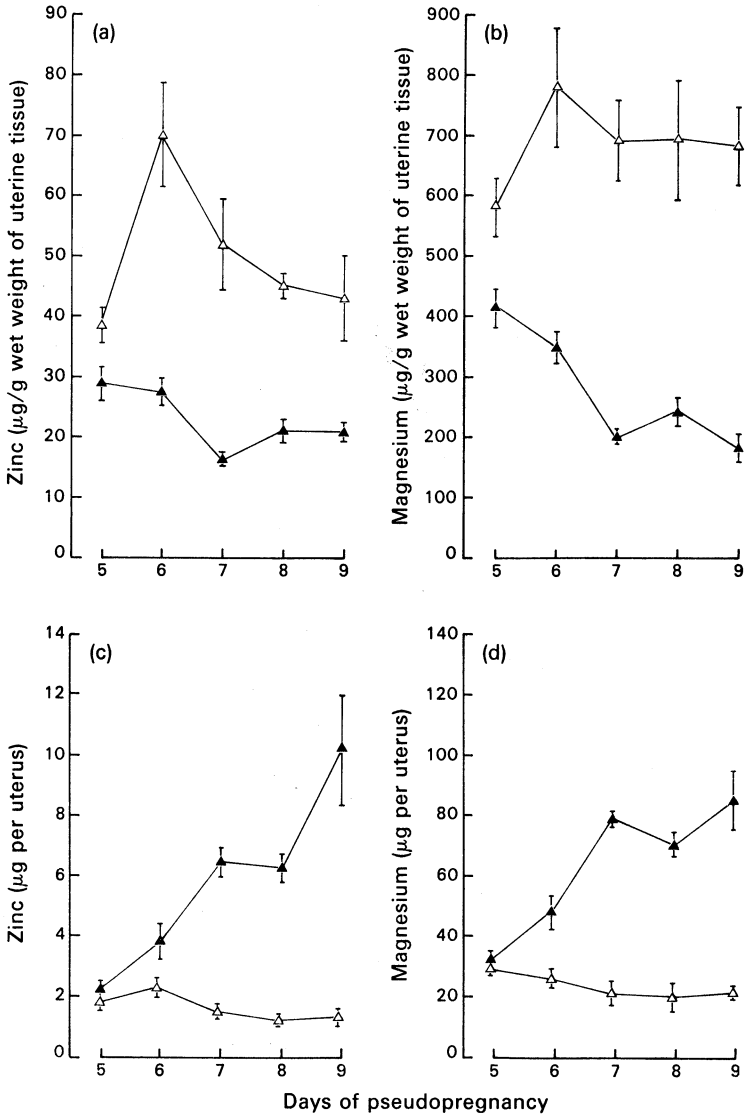
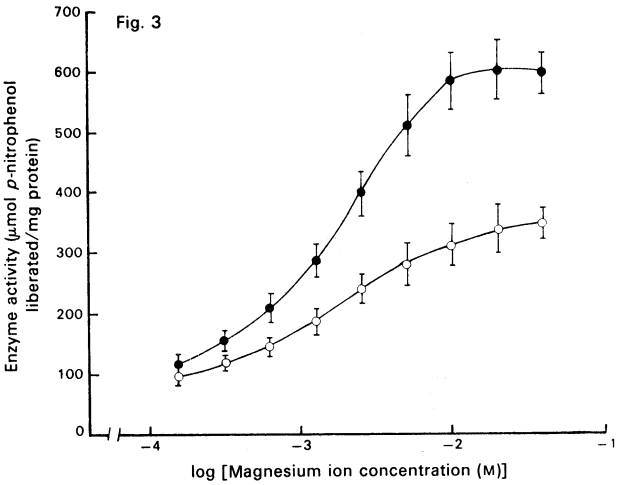


Fig. 2. Concentrations of zinc (*a*) and magnesium (*b*) and total zinc (*c*) and magnesium (*d*) content in uterine horns of mice during pseudopregnancy. An oil injection was given on day 4 in the left uterine horn (▲) to induce a decidual reaction; the right uterine horn (△) was not stimulated. The multiple-range test gave the following ranking (at the 5% level) on the various days of pseudopregnancy for the metal concentrations and content:

Left decidualized horn	Right non-decidualized horn
Zinc concn: 5 = 6 > 8 = 9 > 7	Zinc concn: 6 > 7 > 5; 7 = 8 = 9; 9 = 5
Magnesium concn: 5 = 6 > 7 = 8 = 9	Magnesium concn: 6 > 5; 6 = 7 = 8 = 9; 5 = 7 = 8 = 9
Zinc content: 9 > 8 = 7 > 6 > 5	Zinc content: 9 = 8 = 7 = 6 = 5
Magnesium content: 9 = 8 = 7 > 6 > 5	Magnesium content: 9 = 8 = 7 = 6 = 5

uterine horns than in the decidualized (left) uterine horns of mice between days 5 and 9 of pseudopregnancy (Figs 2*a* and 2*b*). In non-decidualized horns the concentration of both metals was maximal on day 6 of pseudopregnancy, while in decidualized horns metal concentration decreased from a maximum on day 5 to lower levels with advancing pseudopregnancy.



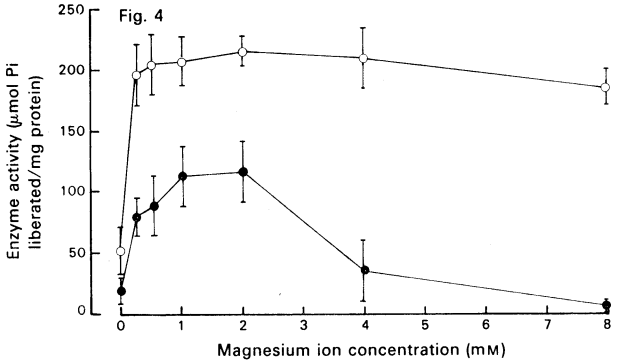


Fig. 3. Effects of Mg^{2+} concentration on the activity of mouse uterine alkaline phosphatase in glycine–NaOH buffer (○) and carbonate–bicarbonate buffer (●).

Fig. 4. Effects of Mg^{2+} concentration on the orthophosphatase (○) and pyrophosphatase (●) activities of mouse uterine alkaline phosphatase. Orthophosphatase activity was assayed with *p*-nitrophenyl phosphate as substrate while pyrophosphatase activity was assayed with ATP as substrate.

Again, when these data were expressed as total μg of metal per uterine horn (Figs 2*c* and 2*d*), the patterns differed from those described above. The total zinc and magnesium content of each decidualized (left) uterine horn was significantly greater ($P < 0{\cdot}01$) than that of non-decidualized (right) uterine horns. The difference increased with advancing pseudopregnancy because increasing amounts of both metals accumulated in decidualized horns while the zinc and magnesium content of non-decidualized horns remained essentially unchanged between days 5 and 9.

In agreement with the data obtained from the pregnancy studies, there was usually about 10-fold more magnesium than zinc in the uterine tissue during pseudopregnancy.

Effects of Mg^{2+} on Alkaline Phosphatase Activity

Fig. 3 shows that the activity of purified mouse uterine alkaline phosphatase increased with increasing concentrations of $MgCl_2$ up to 4·0 mM. The stimulatory effect of $MgCl_2$ was, however, influenced by the buffer used and was significantly greater ($P < 0{\cdot}01$) in carbonate–bicarbonate buffer than in glycine–NaOH buffer.

K_m values (results not shown) for the enzyme hydrolysing various phosphomonoesters were not significantly affected by the buffer used or by Mg^{2+} ions and were similar to those reported previously (Murdoch *et al.* 1980). The value of the energy of activation (49·7 $\pm$ 2·4 kJ mol^{-1}) calculated from linear Arrhenius plots and the response of the enzyme to temperatures between 40 and 60°C were also not affected by the buffer used or the presence of Mg^{2+} ions and the results were again similar to those described previously (Murdoch *et al.* 1980).

Both native and Zn-apoenzyme preparations were stable at temperatures of 0·5 and 37°C for 72 h in 32 mM Tris–HCl buffer, pH 8·0 (results not shown). However, while apoenzyme preparations were also stable over the 72 h period at 0·5°C, their capacity to be reactivated by the addition of Zn^{2+} and Mg^{2+} ions was completely lost after only 8 h at 37°C.

Finally, Fig. 4 shows that the pyrophosphatase activity of mouse uterine alkaline phosphatase, incubated in glycine–NaOH buffer with ATP as substrate, was increased in the presence of 2 mM $MgCl_2$ and decreased with increasing concentrations of the cation. Orthophosphatase activity of the enzyme, however, was stimulated to a greater extent by 2 mM $MgCl_2$ in the presence of *p*-nitrophenyl phosphate, and increasing concentrations of Mg^{2+}, in this case, failed to alter activity.

Discussion

The results of the present study clearly demonstrate that the levels of zinc and magnesium in mouse uterine tissue are influenced by reproductive status and change significantly during early pregnancy and pseudopregnancy. It is also apparent from the results that, while the uterus has the capacity to accumulate zinc and magnesium between days 5 and 12 of pregnancy and between days 5 and 9 of pseudopregnancy, when decidual cells are present, it does not accumulate the metals at a rate sufficient to match increases in weight (Murdoch *et al.* 1978) and thereby maintain constant concentrations in the tissue. The accumulation of the metals appears mainly to be a function of the decidual cells because non-decidualized horns of pseudopregnant mice failed to increase their total content of zinc and magnesium between days 5 and 9. If accumulation of metals in the uterus of this species was simply a function of circulating hormones, similar changes in metal content would be expected in both decidualized and non-decidualized horns. The extent to which alterations in the circulatory system (Young 1956), uterine tissue permeability (Finn and McLaren 1967), or shifting body deposits of zinc and magnesium (Lutwak-Mann and McIntosh 1969) contribute towards the changing uterine metal levels during pregnancy and pseudopregnancy is uncertain at this stage.

The ability of Mg^{2+} ions to stimulate the activity of purified preparations of mouse uterine alkaline phosphatase without influencing K_m values or the response

of the enzyme to changes in temperature is consistent with observations on phosphatases from other sources (Murdoch 1971; Brunel and Cathala 1973; Linden *et al.* 1977; Jung and Pergande 1979). In addition, like other alkaline phosphatases (Ackerman and Ahlers 1976; Chlebowski *et al.* 1979), the uterine enzyme requires zinc to maintain structural stability (present study). Although these effects of zinc and magnesium on alkaline phosphatase make it tempting to suggest that the changing levels of the metals in the uterus during early pregnancy and pseudopregnancy are involved in the regulation of the uterine enzyme, such a proposal may not in fact be valid. Our recent work on mouse uterine alkaline phosphatase (Murdoch *et al.* 1980) has shown that about 8 μg of purified enzyme protein can be obtained from 1 g of day 7 pregnant tissue, representing a yield of 17% recoverable activity. Since the enzyme was also shown to have a molecular weight of about 205000, it can be calculated that approximately $2{\cdot}3 \times 10^{-3}$ μmol of alkaline phosphatase may be present in each gram of mouse uterine tissue on day 7 of pregnancy. In the class of mammalian alkaline phosphatases, like the mouse uterine enzyme (Murdoch *et al.* 1980), that needs both zinc and magnesium for activity, it is probable that 4 mol of Zn^{2+} and either 1 or 2 mol of Mg^{2+} bind to each mole of dimeric enzyme (see Cathala *et al.* 1975). From the results of the present investigation it is evident that if mouse uterine alkaline phosphatase has similar binding affinities, during normal pregnancy and pseudopregnancy the metals would always be present in amounts considerably greater than those required to saturate the enzyme for full catalytic activity. Therefore it is considered that, unless mechanisms exist in the mouse uterus to effect compartmentation and establish concentration gradients of both metals (Gerbitz 1977), zinc and magnesium may not be major factors involved in the regulation of uterine alkaline phosphatase activity during early pregnancy. PetitClerc and Fecteau (1977), however, have proposed that the metals may regulate alkaline phosphatase in the rat placenta.

The different activity responses of alkaline phosphatase to Mg^{2+} ions in different buffer systems emphasize the need to carefully standardize reaction conditions during studies of phosphatase enzymes. Although buffer effects of a similar nature have been described by other investigators (see Rej 1977), satisfactory reasons to explain them have not been found (Ghosh *et al.* 1977). The effects of Mg^{2+} ions on the orthophosphatase and pyrophosphatase activities of the uterine enzyme are also similar to those described for other phosphatases (Murdoch 1971; Seargeant and Stinson 1979). These observations support the view that pyrophosphatase behaviour is activated by low concentrations of Mg^{2+} when the complex ion $MgP_2O_7^{2-}$ is available as substrate but is progressively inhibited as the Mg^{2+} to pyrophosphate concentration ratio is increased and a $Mg_2P_2O_7$ complex is formed (see Nayudu and Miles 1969).

In conclusion, the results of the present study show that zinc and magnesium levels in the uterus of the mouse change significantly during early pregnancy and pseudopregnancy. While it has also been shown that the metals exert major effects on the orthophosphatase and pyrophosphatase activities and stability of the uterine metalloenzyme alkaline phosphatase *in vitro*, the changes in the uterine content of zinc and magnesium are not considered to be major factors involved in the *in utero* regulation of the enzyme during early pregnancy. Studies are now in progress to assess the function of the uterine genome during decidualization to determine its involve-

ment in the regulation of alkaline phosphatase by directing changes in the rate of *de novo* synthesis of the enzyme.

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