vesicles were produced either from pupae, or from newly hatched imagoes. For studying the effect of hormonal deficiency on the development of the oocyte, 2nd-day pupae were deprived of brain, of corpora cardiaca, corpora allata and prothoracic glands by cutting off the anterior part of the body after the technique of WILLIAMS[1]. The ovarioles were dissected from the isolated abdomina obtained by this technique on the 5th day after the operation, that is on the day which for normally developing pupae is the second last day of pupal life. Normally, at that time the terminal egg vesicles of the ovarioles contain fully developed oocytes[2].

The tissue was dissected without narcosis, fixed in glutaraldehyde in cacodylate buffer at pH 7.3 and embedded in Epon 812[3]. The material was sectioned on a Reichert ultramicrotome and subsequently contrasted with uranyl acetate followed by lead citrate[4,5] and examined in the JEM 7A electron microscope.

*Results and discussion.* In the terminal egg vesicles found in pupae of *G. mellonella* at the 6th day after pupation, that is at the onset of vitellogenesis[2], numerous protrusions of the plasma membrane extending to the cortical zone of the oocyte are conspicuous. Similar protrusions were observed in oocytes of several other insects[6–8]. However, in contrast to what was found in the egg vesicles of *Hyalophora*, no special microvilli in the apical part of the follicle cells and no brush border on the oocyte surface were observed. In *Galleria*, the pattern of both the follicular cell and the oocyte plasmalemma may be easily followed (Figure 1). These two membranes seem to be in close contact with one another, the distance between them measuring about 150 Å, when not filled with an amorphous adielectronic material. The agglomeration of this material is accompanied by local extension of the intercellular space. Numerous slender plasma membrane invaginations appear, which are partially filled with a substance of similar electron density as that found in the intercellular space. The invaginations seem to be formed by the oocyte plasmalemma only, indicating high pinocytotic activity of this membrane (Figure 2) similarly as was found in *Colorado beetle* egg vesicles[9].

In the course of vitellogenesis, the follicular/oocyte interspace becomes gradually more extended and nearly uniformly filled with electron dense material.

The developing oocyte in the course of vitellogenesis is provided with proteins[10,11] and probably lipids[12] produced by the fat body and transported by the haemolymph. However, the follicle cells themselves also provide the developing oocyte with newly synthetized lipids[13,14], proteins and glycoproteins[15]. Some of these components are most probably used for the formation of the vitelline membrane, which, as has been found in the oocytes of *Drosophila*, contains lipids, proteins and polysaccharides[16]. This chemical heterogeneity of the vitelline membrane is reflected in the observed non-homogeneity of its ultrastructural pattern (Figures 3 and 4). The material accumulating at the oocyte border and forming the membrane is not of equal electron density, which is especially distinct at the first stages of the membrane formation. It is worth noting that, even in very advanced stages of vitellogenesis, the vitelline membrane retains its spongy ultrastructure, which probably enables it to continue the pinocytotic activity. Even when is it about 1 μm thick, the delicate invaginations of the oocyte plasmalemma ending with pinocytotic vesicles are seen.

It is known that cutting off the normal hormone inflow disturbs the normal development of the egg vesicles of *G. mellonella*. Light microscopic investigations showed that oocytes growing in isolated abdomina do not initiate vitellogenesis and reach not more advanced stages than those found in 4-day-old normal pupae[17].

The EM study shows that development stops even at an earlier stage, since, contrary to the terminal oocytes of 4th-day pupae, the plasmalemma of terminal oocytes of experimental individuals fails to show the pinocytotic activity. The external epithelial sheath of these egg vesicles is distinctly enlarged, as well as the follicular epithelium which even becomes multilayered. However, the contact of the apical part of the follicle cells with the oocyte seems to be rather loose and no accumulation of material in the interfollicular cell spaces and at the oocyte/follicle interface can be observed (Figures 5 and 6).

It seems probable that the lack of the pinocytotic activity of oocyte plasmalemma in ovarioles developing in hormonal deficiency is caused by the lack of nutritive materials provided to the oocytes. This may be the consequence as well of the diminution of synthetic activity in follicle cells, as of inhibition of production or release of nutritive material from the fat body involved by the experiment[18].

*Résumé.* Le plasmalemme des oocytes se caractérise par une grande activité pinocytosique au cours de la vitellogenèse. Celle-ci semble être contrôlée par des facteurs hormonaux, car l'ablation des glandes endocrines bloque complètement cette activité.

A. PRZEŁECKA and A. B. DUTKOWSKI

*M. Nencki Institute of Experimental Biology, Department of Cell Biology, Polish Academy of Science, Warszawa (Poland), 3 May 1971.*

1 C. M. WILLIAMS, Biol. Bull. *93*, 89 (1947).
2 A. DUTKOWSKI, Zoologica Pol. *19*, 115 (1969).
3 J. H. LUFT, J. biophys. biochem. Cytol. *9*, 409 (1961).
4 H. E. HUXLEY and G. ZUBAY, J. biophys. biochem. Cytol. *11*, 273 (1961).
5 E. S. REYNOLDS, J. Cell. Biol. *17*, 208 (1963).
6 E. ANDERSON, J. Cell Biol. *20*, 131 (1964).
7 C. FAVARD-SERÉNO, J. Microsc. *3*, 323 (1964).
8 T. F. ROTH and K. R. PORTER, J. Cell Biol. *20*, 313 (1964).
9 A. DE LOOF and A. J. LAGASSE, J. Insect Physiol. *16*, 211 (1970).
10 W. H. TELFER, J. gen. Physiol. *37*, 539 (1954).
11 B. STAY, J. Cell Biol. *26*, 49 (1965).
12 A. DUTKOWSKI and B. ZIAJKA, Zoologica Pol. *20*, 55 (1970).
13 A. PRZEŁECKA, Ann. Histochim. *11*, 403 (1966).
14 A. PRZEŁĘCKA and A. DUTKOWSKI, Bull. Acad. pol. Sci. *13*, 573 (1965).
15 L. M. ANDERSON and W. H. TELFER, Tissue Cell *1*, 633 (1969).
16 R. C. KING, Growth *24*, 265 (1960).
17 A. DUTKOWSKI and A. PRZEŁĘCKA, Zoologica Pol. *19*, 151 (1969).
18 This work was supported in part by Grant No. FG-Po-221 from the U.S. Agricultural Department.

## Reduced Foetal Calcium Without Skeletal Malformations in Rats Following High Maternal Doses of a Strontium Salt

Skeletal malformations have been reported following high maternal doses of 'bone-seeking' metal ions such as lead[1,2], cadmium[2–4], zinc[3] and lithium[5–7] to laboratory mammals. The teratogenic activity of strontium, a metal ion thought to be essential in trace amounts for initial osteogenesis[8], has not been studied. In mature animals,

this ion is absorbed to the hydroxyapatite matrix of bone or is chemically incorporated into the mineral phase as a non-diffusible phosphate[9]. The present investigation into the effects of high maternal doses of a stable strontium salt given during the period of maximal foetal osteogenesis would seem important in view of previous work in which high concentrations of strontium ion have been shown to inhibit calcification in the bone of neo-natal mice[10] and in bone cultured in vitro[11].

Virgin female rats of the Wistar strain were mated with proven males and the day when spermatozoa were first detected in vaginal smears was designated day 0 of pregnancy. The animals were given a standard diet containing 0.9% calcium and negligible quantities of strontium. Distilled water was allowed ad libitum. Groups of 3 females were treated subcutaneously with doses of 25, 50, 100 or 200 mg/kg strontium nitrate in 1 ml of distilled water from days 9 to 19 of pregnancy when they were killed with an overdose of pentobarbitone sodium (Nembutal). Control animals were given 1 ml of distilled water subcutaneously. The foetuses were preserved in 10% buffered formalin for histology or in 95% ethanol for potash maceration and alizarin staining.

Histological sections of foetuses cut transversely through the cephalic, thoracic and lumbar regions were stained with haematoxylin and eosin, haematoxylin and van Geisen, toluidine blue with silver impregnation[12] and the sodium rhodizonate method for the presence of strontium[13].

The progeny from the treated mothers did not differ from the controls in size or body weight. The litter sizes were similar and the number of resorption sites was not increased (Table I). In the alizarin stained foetuses, the skeletons were normal and the zones of calcification in the bones were of normal proportions. No histological changes were detected in the soft tissues and the skeletal tissues exhibited the characteristic degree of ossification for 19-day-old rat foetuses. Sodium rhodizonate staining for strontium ion was very pale and only observed in ossifying regions and not in soft tissues. These results indicate that under the conventional methods of investigation high maternal doses of strontium nitrate are not teratogenic when given at a maximum period of bone deve. lopment.

To ascertain the levels of strontium ion actually accumulating in the foetuses, 3 foetuses from mothers given 50 and 200 mg/kg strontium nitrate were ashed and the residue dissolved in 6*N* hydrochloric acid. The solutions were then analyzed by atomic absorption spectrophotometry for their calcium and strontium contents. The results are given in Table II.

These results show that the level of strontium in the foetuses is unchanged despite a four-fold increase in the maternal dose of the strontium salt. A state of 'saturation' would seem to be reached in the foetuses at doses of 50 mg/kg, when the calcium:strontium ratio attained is approximately 40:1. This is in agreement with a previous observation in which a saturation point is reached in young rats when the ratio is 50:1[14]. These authors considered that the saturation was due to a maximal adsorption of strontium to the osteoid protein, a mechanism by which most of the initial ion uptake occurs. Limited strontium uptake after this point is possible by incorporation of the ion into the hydroxyapatite structure.

In contrast to the constant level of foetal strontium, the foetal calcium from mothers given 200 mg/kg was appreciably less than in those given 50 mg/kg. To account for this observation, one may speculate that the high level of maternal strontium either antagonizes the transplacental passage of calcium or alternatively that the strontium ion upsets the maternal-foetal calcium balance such that less calcium is available for the foetuses than usual.

The results of the present study lead to the conclusion that the 'bone-seeking' properties of the metal alone are insufficient to produce skeletal abnormalities. Evidence exists that the 'bone-seeking' metals cadmium, zinc, lead and lithium possess cytotoxic properties when administered at high doses to laboratory animals[9,15–17]. In addition, cadmium damages the chondrocytes of the developing hamster palate[18] while lead has been shown to cause chromosomal damage in leukocyte cultures[19]. It would thus seem that the skeletal abnormalities produced by these and other 'bone-seeking' metals may result from some funda-

Table I. The total numbers of foetuses and resorption sites in rats given subcutaneous doses of strontium nitrate in pregnancy

| Dosage (mg/kg) | Animals treated | Total No. of foetuses | Total No. of resorptions |
|---|---|---|---|
| Control | 3 | 36 | 3 |
| 25 | 3 | 36 | 6 |
| 50 | 3 | 30 | 2 |
| 100 | 3 | 31 | 4 |
| 200 | 3 | 33 | 2 |

Table II. Strontium and calcium content of foetuses from mothers given strontium nitrate in pregnancy

| Dosage (mg/kg) | No. of foetuses | Strontium content (ppm) | Calcium content (ppm) | Ratio Sr:Ca |
|---|---|---|---|---|
| 50 | 3 | 5.4 ± 0.1 | 215 ± 15 | 1:40 |
| 200 | 3 | 4.6 ± 0.5 | 82 ± 18 | 1:18 |


[1] V. H. Ferm and S. J. Carpenter, Expl molec. Path. *7*, 208 (1967).
[2] V. H. Ferm, Experientia *25*, 26 (1969).
[3] V. H. Ferm and S. J. Carpenter, Lab. Invest. *18*, 429 (1968).
[4] R. E. Holmberg and V. H. Ferm, Arch. envir. Hlth. *18*, 873 (1969).
[5] H. Fries, Lancet *1*, 1233 (1970).
[6] K. T. Szabo, Lancet *2*, 849 (1969).
[7] K. T. Szabo, Nature, Lond. *225*, 73 (1970).
[8] O. Rygh, Bull. Soc. Chim. biol. *31*, 1052 (1949).
[9] E. Browning, in *The Toxicity of Industrial Metals* (Butterworth, London 1961).
[10] N. S. MacDonald, F. Ezmirlian, P. Spain and C. MacArthur, J. biol. Chem. *189*, 387 (1951).
[11] F. W. Lengemann, Proc. Soc. exp. Biol. Med. *94*, 64 (1957).
[12] A. B. G. Lansdown, Histochemie *13*, 192 (1968).
[13] D. F. Waterhouse, Nature, Lond. *167*, 358 (1951).
[14] N. S. MacDonald, R. E. Nusbaum, R. Stearns, E. Ezmirlian, C. MacArthur and P. Spain, J. biol. Chem. *188*, 137 (1951).
[15] S. A. Gunn, T. C. Gould and W. A. D. Anderson, J. Reprod. Fert. *21*, 443 (1970).
[16] S. A. Gunn, T. C. Gould and W. A. D. Anderson, Biol. Reprod. *3*, 35 (1970).
[17] V. Totović, Virchows Arch. path. Anat. Physiol. *339*, 151 (1965).
[18] J. E. Mulvihill, S. H. Gamm and V. H. Ferm, J. Embryol. exp. Morph. *24*, 393 (1970).
[19] L. A. Muro and R. A. Goyer, Arch. Path. *87*, 660 (1969).

mental interference in the function of chondrocytes and osteocytes rather than by their mere deposition in bone.

*Zusammenfassung.* Nachweis, dass hohe Strontiumkonzentrationen die Kalzifikation der Knochen neugeborener Mäuse und diejenige in Knochengewebskulturen hemmen. Im Gegensatz dazu und zu anderen knochenaffinen Metallen wirkt Strontiumnitrat in Dosen von 25, 50, 100 and 200 mg/kg, verabreicht während des 9. bis 19. Trächtigkeitstages, bei Ratten nicht teratogen.

[20] We are indebted to Mr. K. L. MURRAY of the Government Chemist's Laboratory for analyzing tissue samples by atomic absorption spectrophotometry. We are also grateful to Dr. R. F. CRAMPTON, Director of BIBRA, for his interest and advice in this work.

A. B. G. LANSDOWN, R. C. LONGLAND and P. GRASSO[20]

*The British Industrial Biological Research Association, Woodmansterne Road, Carshalton (Surrey, England), 25 October 1971.*

## Esterases in the Hearts, Lungs and Livers of Human Foetuses Before Mid-Term

BLANCO and ZINKHAM[1] have presented the non-specific esterase zymograms of various human tissues including some from foetal specimens of advanced gestational age (5 months and onwards). All tissues contained multiple bands of esterase activity in patterns which increased in complexity as development proceeded. Variations in pattern between different tissues were observed at all ages and their findings are comparable with those of MARKERT and HUNTER[2] who studied the mouse. However, PAUL and FOTTRELL[3] were unable to demonstrate any differences between the esterases in human tissues of either adult or foetal origin.

This paper presents evidence for the existence of esterases in much younger human foetuses and demonstrates the changes that take place in the period of gestation prior to that examined by BLANCO and ZINKHAM.

Ten normal human foetuses obtained at hysterotomy were used in this study. Their crown rump lengths varied between 40 and 160 mm. Both lyo and desmo tissue extracts were prepared as described earlier[4]. Starch gel electrophoresis of all specimens was carried out at pH 7.5 with a horizontal run at 4°C for 4 h employing a constant current with an average voltage drop of 12 volts/cm.

Simultaneous coupling azo dye reactions were performed at pH 7.5 using α-naphthyl acetate and proprionate as substrates (0.25 mg/ml) with Fast Blue 2B (1 mg/ml) as the diazonium salt in each case. The effects of the inhibitors eserine and E600 (Diethyl 4-nitro phenylphosphate) were both studied at molar concentrations of $1 \times 10^{-5} M$. Mipafox [*bis* (isopropylamine) phosphinocofluoridate] was employed at $2 \times 10^{-5} M$.

Esteraseswere demonstrated in all the specimens so examined. The desmo fraction exhibited fewer and less prominent bands than the lyo fraction. However, all these 'desmo' bands were represented in the 'lyo' zymogram and responded similarly to inhibition. The progression in complexity may be divided into 2 stages, firstly at 40–80 mm and secondly, from 100 to 160 mm crown rump length. At the 40 mm stage each of the 3 organs studied has a quite specific zymogram pattern (Figures 1, 2 and 3). With α-naphthyl acetate as substrate a total of 4 isozymes are present (A, B, C and D). Some of the isozymes are sensitive to treatment with E600 (Figure 1). The response of any one band to inhibition may, however, differ from one organ to the next, for example, the bottom band C of activity in the lung (Figure 3) as visualized with the α-naphthyl acetate substrate shows no inhibition by E600. In contrast, the equivalent band C in the liver (Figure 2) is completely inhibited by this compound using the same substrate.

Three isozymes A, B and C are found when the α-naphthyl proprionate substrate is employed. The two most slowly migrating bands A and B are both insensitive to E600 in all 3 tissues whereas the bottom band C is consistently inhibited. An individual band may however manifest differential sensitivity to E600 with different substrates. The bottom band C in the lung (Figure 3) is not inhibited by E600 using α-naphthyl acetate but is inhibited when the

[1] A. BLANCO and W. H. ZINKHAM, Bull. John Hopkins Hosp. *118*, 27 (1966).
[2] C. L. MARKERT and R. L. HUNTER, J. Histochem. Cytochem. *7*, 42 (1959).
[3] T. PAUL and P. FOTTRELL, Biochem. J. *78*, 418 (1961).
[4] I. M. TAYLOR and R. B. SMITH, J. Histochem. Cytochem. *19*, 498 (1971).

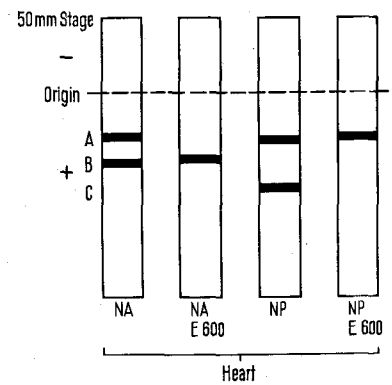


Fig. 1. 50 mm Human heart. Zymogram of 'lyo' extract showing 3 isozymes A, B and C. α-naphthyl acetate (NA) and propionate (NP) were employed as substrates.

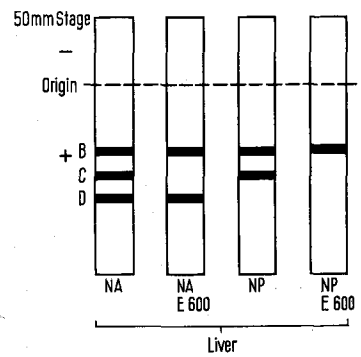


Fig. 2. 50 mm Human liver. Zymogram of 'lyo' extract containing 3 isozymes B, C and D.