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INSULIN INDUCED MICROMELIA IN CHICKENS

II. *Biochemical Study*

By

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The mechanism by which the different teratogenic agents induce congenital malformations is largely unknown. In the case of insulin-induced generalized skeletal deformities in chickens, very little is known as to whether there is any response of the teratogenic influence on the biochemical pattern of the skeleton. However, it may be assumed that the course of the abnormal embryonic development is to some extent reflected in the chemical composition of the bone. Furthermore, a correlation might exist between the morphological, histological and biochemical development of the skeleton. If such internal relationship can be proved, a better understanding of the mechanism by which the congenital malformations are induced, can be expected.

Works concerning the biochemical composition of the normal skeleton of the chicken during the embryonic development are very few. Similarly, biochemical studies of the skeleton during the embryonic development of insulin-induced micromelia are extremely rare.

Determination of certain chemical components of the embryonic chicken tibia has been performed partly in normal, partly in insulin-induced micromelic embryos and the results are reported in this paper. Furthermore, the results obtained here are compared and discussed in relation to the morphological and histological observations made on the same material, which have been reported in an earlier publication (*Sevastikoglou*, 1963 a).

The material and the methods used for the collection of normal and insulin-induced micromelic bone are the same as in the previous publication. The biochemical studies reported here are based on quantitative determinations of the alkaline and acid phosphates activities and the hydroxyproline content of the whole tibiae. For determination of the

alkaline and acid phosphatase activities the methods of *King & Armstrong* (1934) as modified by *Sevastikoglou* (1958), was used. Hydroxyproline determinations were performed by the method described by *Newman & Logan* (1950).

RESULTS

The alkaline phosphatase activity of the normal bone was very low and constant between the 5th and 7th days of incubation. It rose between the 7th and 9th days, and thereafter almost constant values were obtained up to the 12th of incubation. After this time a very steep rise was observed up to the 16th day, and this was followed by a fall at the 17th and 18th days of incubation. The alkaline phosphatase activity in

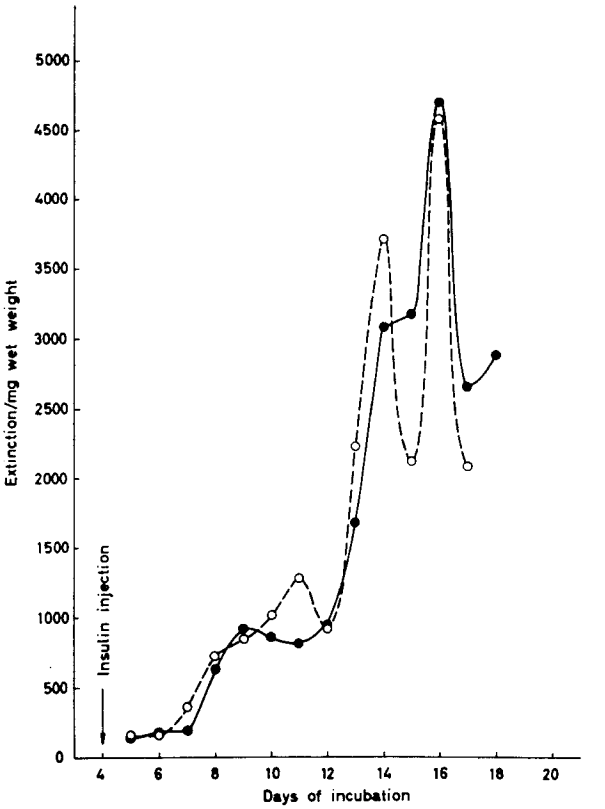

Fig. 1.

The alkaline phosphatase activity of the tibia in normal (continuous line) and insulin-injected (broken line) embryos during the embryonic development.

the insulin-induced micromelic bone followed a curve, almost parallel with that of the normal bone between the 5th and the 9th days of incubation. The rise of the enzymatic activity after this period showed an undulating development with deep waves which, however, showed almost the same tendency to rise as in the normal bone. The first apparent difference between the alkaline phosphatase activity between normal and insulin-injected material appeared after the 10th day of incubation. (Fig. 1).

The acid phosphatase activity both in the normal and the insulin-induced micromelic bone showed an irregular undulating development during the course of the incubation. In the micromelic bone, however, the waves of the curve were somewhat higher and sharper than in the curve of normal bone. (Fig. 2).

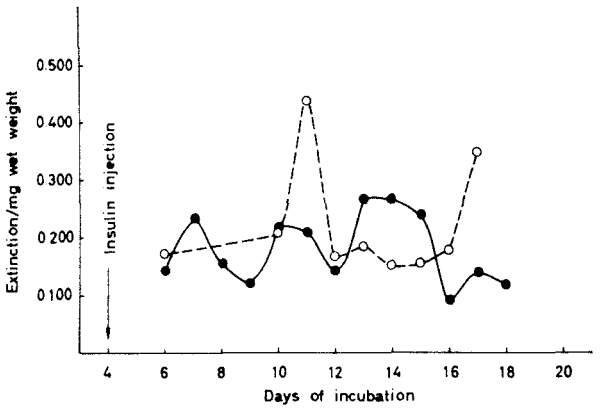

Fig. 2.

The acid phosphatase activity of the tibia in normal (continuous line) and insulin-injected (broken line) embryos during the embryonic development.

The hydroxyprolin content of the tibiae of the normal embryos was extremely low up to the 6th day of incubation. A more or less regular and steep rise was observed consistently up to the 20th day. An almost similar curve was formed by the values of the micromelic tibiae between the 5th and 15th days of incubation. The values obtained were, however, in the majority of the cases lower than the respective values for normal material. The difference in the values was larger between the 7th and 9th and between the 12th and 13th days of incubation, while, the 10th and 15th day values were almost exactly the same. (Fig. 3).

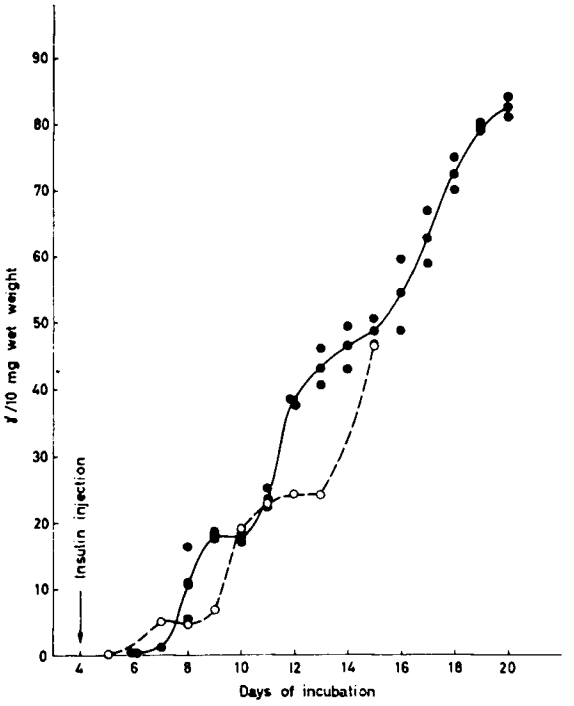

Fig. 3.
The hydroxyprolin content of the tibia in normal (continuous line) and insulin-injected (broken line) embryo during the embryonic development.

DISCUSSION

The nature of the investigation and the difficulty in obtaining sufficient values made it impossible to analyse the results reported in this part of the work statistically. The study of the alkaline and acid phosphatase activity and the collagen content of the tibiae of normal and insulin-injected material during the embryonic development provide, however, evidence that there are differences in the biochemical reactions of the bone in these two groups of material. In their work *Anderson and co-workers* (1959) reported that there were no marked changes in the chemical composition of the cartilage cell or its matrix in insulin dwarfism. The results of these authors are, however, based on estimations of certain substances (glycogen, alkaline phosphatase and phosphorylase activity and chondroitin sulphate) on only the cartilaginous parts of the long bones of the lower extremities, of normal and insulin-treated embryos, 14 days after the injection of insulin into the egg, i.e.

at the 18th day of incubation. Moreover, their results are almost exclusively based on histochemical methods, the value of which for quantitative estimation is questionable.

The evolution of the alkaline phosphatase activity of the normal tibiae during the embryonic development showed a great similarity to the evolution of the enzymatic activity in the cartilaginous cones of normal embryos, as previously observed (*Sevastikoglou*, 1958). The enzymatic activity increased, however, in the case of the tibiae earlier than in the cones and it showed a decrease at the later stages of embryonic life. The role of this enzyme on the normal osteogenesis is not yet known completely and it has been suggested that alkaline phosphatase is engaged in cell differentiation in general and in the differentiation of the osteoblasts especially in the case of osteogenesis (*Kabat & Furth*, 1941, *Moog*, 1943 and -44, *Sevastikoglou*, 1958 and others). The divergences in the alkaline phosphatase activity observed between normal and insulin-treated tibiae in this work appeared first after the 10th day of incubation. During the normal embryonic development of the chick, osteoblasts differentiate from the periosteum of the long bones and lay down bone at about the same time of incubation. The results obtained here provide more evidence for the participation of this enzyme in the process of osteoblast differentiation. Furthermore, they provide more evidence for the suggestion made earlier (*Sevastikoglou*, 1963 a) that insulin affects the process of osteogenesis itself, which starts at about the 9th day of incubation.

Acid phosphatase has only previously on very few occasions been studied in connection with osteogenesis (*Castellá*, 1952, *Tonna*, 1957, *Sevastikoglou*, 1958). The role played by this enzyme in this process is quite unknown. It has been suggested earlier that a relation might exist between acid phosphatase activity and bone matrix formation (*Sevastikoglou*, 1958). As in the case of alkaline phosphatase, study of the acid phosphatase activity in the present work showed deviations between normal and insulin-treated bone first after about the 10th day of incubation, providing more evidence of the role of insulin in the induction of the skeletal deformities.

A point which has been the object of special study was the question of whether there was any difference in the alkaline and acid phosphatase activity between the two types of insulin-induced skeletal deformities described earlier (*Sevastikoglou*, 1963 a). Special estimations of the enzymatic activities in micromelic tibiae without other manifest deformities and in chondrodystrophic tibiae at the 15th day of incuba-

tion showed no significant differences in the alkaline phosphatase activity. However, acid phosphatase activity was higher in the chondrodystrophic than in the micromelic bone. Because of the unknown role played by the acid phosphatase it is impossible to comment on the value of this observation.

The hydroxyproline content of the tibiae indicated their collagen content. Hydroxyproline determinations of normal and insulin-treated tibiae showed that there was a difference in the values obtained among the bones of the two groups especially marked between 7th and 9th and between 12th and 13th days of incubation. These differences might indicate that the collagen content of the insulin-treated bones showed a decrease already before micromelia had appeared and after a normalisation of the values at the 9th day a new decline appeared between the 10th and 13th days of incubation. The former deviation from the normal is difficult to interpret. The latter, however, might depend on the insulin effect of osteogenesis mentioned previously.

The results of this part of the work indciate that there is a connection between the morphological changes described earlier (*Sevastikoglou*, 1963 a) and the biochemical response of the skeleton under the teratogenetic influence of insulin. Moreover, evidence has been accumulated both from morphological and biochemical studies that the abnormal reaction of the skeleton appears first long after the injection of insulin and when the osteogenetic process itself has begun to develop, i.e. the 9th day of incubation of thereabout.

The way in which insulin induces congenital abnormalities in the hen embryo has been the subject of several investigations and extensive discussion. It is more likely that this hormone produces alteration in metabolism of the embryo and that different tissues have a specific susceptibility towards the noxious influence of insulin during a certain particularly critical period of their development during embryonic life. Several experimental works have shown that insulin influences the normal development of the chick by upsetting the carbohydrate metabolism of the embryo. *Hanan* (1925), *Dalton & Hanzal* (1940), *Zwilling* 1948, and *Duraiswami* (1952) by estimations of the blood sugar in treated embryos found that hypoglacaemia occurs after injection of insulin during the early stages of embryonic development. Lowered blood sugar values persist until the 12th day of embryonic life, and the embryos regain normal blood sugar levels by the 14th day of incubation (*Zwilling*, 1948). *Zwilling* also observed that embryos with a marked hypoglycaemia up to the 12th day of incubation showed the most ex-

treme skeletal deformities. If sugar levels returned to normal between the 8th and 10th days, the embryos developed only moderate micromelia. The embryos which showed recovery of the normal blood sugar levels by the 8th day of incubation exhibited no deformity at all and were indistinguishable from normal embryos. In an attempt to mitigate insulin micromelia by adding glucose, *Zwilling* (1952) found that glucose seemed to exaggerate the effect of insulin. *Landauer* (1946) and *Zwilling* (1949) found that the morphological affect of insulin could be prevented by simultaneous administration of nicotinamide. According to *Zwilling* (1952), these data show that disturbances of the carbohydrate cycle, at some point, are involved in the production of the anomalies by insulin. The author states further (1948) that apparently some mechanism of sugar control which becomes effective some time between the 12th and 14th days of incubation overcomes the hypoglycaemic effects of insulin. This statement according to *Duraiswami* (1952) may be supported by the observations of earlier investigators (*Idzumi*, 1924, and *Portrin & Aron*, 1927) that the embryonic liver of the chick begins to store glycogen in appreciable quantities about the 11th day of incubation and that it is only later that the carbohydrate metabolism in the chick embryo is controlled by interaction between the appropriate endocrine glands, which also begin to secrete active hormones at about the same time. Based on these observations *Duraiswami* (1952) concluded that presumably insulin could induce the various abnormalities before this control is established in the embryo.

Chen (1954) in a study of the insulin effect on chick embryonic, 6–7 day old, bones grown in tissue culture observed structural changes of the bone explants very similar to these observed in vivo under the influence of the hormone and concluded that its action is a very complex one. He further stated that it seems unlikely that insulin affects the production of a single substance, such as chondroitin sulphate, as suggested by *Duraiswami*.

Any discussion of the mechanism by which insulin affects the development of the embryonic skeleton lies beyond the scope of the present investigation. In the light of the results obtained in the present investigation it is, however, evident that insulin injection in fertilized hens' eggs induces abnormal morphological and biochemical reactions of the skeleton, resulting in deformities which first appear at about the 9th day of incubation. At this period of embryonic development bone is laid down in the cartilaginous extremities. It can therefore, be assumed that insulin by some unknown mechanism intervenes directly

in the process of osteogenesis, as for instance by upsetting protein metabolism, as it has been suggested by *Young* (1953). More evidence is required before this hypothesis can be accepted.

SUMMARY

Apart from the morphological changes described in a previous publiaction, insulin injection in fertilized hens' eggs at the 4th day of incubation results in abnormal biochemical reactions of the skeleton.

A correlation between the morphological and biochemical distortions of the skeleton is apparent, appearing first at about the 9th day of incubation.

RESUME

A part certaines altérations morphologiques décrites dans une publication antérieure, l'injection d'insuline dans les oeufs de poules fertilisés, le 4ème jour de l'incubation, donne des réactions biochimiques anormales du squelette.

La corrélation entre ces altérations morpholiques et biochimiques du squelette est évidente, n'apparaissant que le 9ème jour de l'incubation.

ZUSAMMENFASSUNG

Abgesehen von den morphologischen Veränderungen, die in einer vorhergehenden Veröffentlichung beschrieben wurden, verursacht die Insulininjektion in befruchtete Hühnereier am 4. Tage der Ausbrütung auch abnorme biochemische Reaktionen des Skelettes.

Eine Wechselwirkung zwischen den morphologischen und biochemischen Veränderungen des Skelettes, die erst ungefähr am 9. Tag der Bebrütung auftreten, ist offenbar.

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