Abnormalities in Offspring of White Rats Given Protamin Zinc Insulin During Pregnancy.* (19084)

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The studies reported here were designed to determine what effects the injection of large doses of protamine insulin in pregnant rats might have on fetal development. They were undertaken as an extension of investigations on the influence of various environmental factors on the occurrence of congenital malformations in the rat that have been described in various reports from this laboratory (1-3).

Interest in the possibility that insulin injected into pregnant animals might have teratogenic effects on fetal development has been stimulated by reports that insulin injected into developing eggs produced malformations in chick embryos. Landauer(4-6) found that insulin injected into the yolk sac led to rumplessness and abnormalities of the beak, extremities and eyes; others have confirmed these findings(7,8). Recently Duraiswami(9) reported the production of gross skeletal abnormalities in developing chicks by injections of crystalline insulin into developing eggs on the first to the sixth days of incubation. Duraiswami found that the growth of the embryo as a whole was retarded to a degree varying with the dose of insulin, and that different parts of the body were affected to varying degrees according to different days when the injections were done. Primary effects on different parts of the body apparently depended on which part or organ was in the most active stage of development at the time of the injection. Ferrill reported (10) that prolonged administration of subshock doses of insulin in rats, 20 to 40 units per kg of body weight per day (presumably unmodified insulin) had no significant effects on reproduction in the first generation or the succeeding 5 generations. The report does not mention malformations.

Methods. The studies were done during the period from March, 1949, to May, 1950, using young albino rats that were reared on a diet known to be adequate for growth and development, rich in B-complex. The females were not mated until they attained a weight of 150 g. Kept in individual cages, they were weighed and vaginal smears were examined daily; when they were found to be in oestrus, males were placed with them. The day on which sperm were found in the vaginal smear was recorded as the first day of pregnancy, with recognition that this tabulation involved possible errors up to 24 hours in calculating gestation periods. Trials of various dosages of protamin zinc insulin (PZI) were made to determine the maximum amount that would permit survival and good reproduction. Six pregnant rats given 10 units of PZI daily died within 6 days, although non-pregnant rats tolerated considerably higher doses for longer periods. Seven to 8 units of PZI given subcutaneously each day as a single dose appeared to be the maximum amount tolerated.‡ With this dosage, adopted for the experiments, there was a wide variation in mortality rates, ranging from 15 to 75% among different

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7. Couch, J. R., Cravens, W. W., Elvehjem, C. A., and Halpin, J. G., *Poultry Sci.,* 1949, v28, 276.

8. Zwilling, E., *J. Exp. Zool.,* 1948, v109, 197.

9. Duraiswami, P. K., *Brit. Med. J.,* 1950, v2, 384.

10. Ferrill, H. W., *Endocrinology,* 1943, v32, 449.

‡ The insulin was the U-40 solution, supplied by Eli Lilly Co., Indianapolis.

groups and at different times. The highest mortality occurred during the very warm summer of 1949. The insulin was injected daily throughout the gestation period, beginning on the second day of pregnancy or earlier. In a few instances when vaginal bleeding, maternal loss of weight or failure to gain indicated that the fetuses were probably resorbed, the insulin injections were not interrupted but the rat was remated and the injections then continued during a new pregnancy. Injections of insulin before mating did not notably affect the capacity for conception or the course of gestation. A control group of 41 rats received no injections of any sort; but in another investigation conducted at about the same time in the same laboratory, a control series of normal pregnant rats received injections of 0.2 cc of 0.9% solution of NaCl subcutaneously daily or several times a day without apparent effect on gestation. Of 55 rats injected with insulin, 24 received 7 units and 31 received 8 units daily.

The 96 rats included in the study produced (in 96 litters) 783 fetuses. Some rats were allowed to await normal delivery, expected normally on the 23rd day of pregnancy, but most of them were anesthetized and opened on the 22nd day. Before the 22nd day a few rats delivered spontaneously or were opened when signs of probable resorption appeared. A few rats found dead on the 21st day were opened and the dead fetuses were removed and included in the series. None of the latter fetuses showed signs of maceration nor external signs of developmental abnormalities. The fetuses in each litter were counted and in most cases weighed, and then by random selection they were either placed in alcohol for clearing and staining bone by the Schultze-Dawson method(11), or placed in formalin to be fixed for later serial sectioning. The control group of 41 litters included 371 fetuses, of which 176, or 47.4%, were cleared and stained by the Schultze-Dawson method. Fifty-five litters from insulin-treated rats included 417 fetuses, of which 257, or 62.4%, were similarly prepared. The present report concerns only studies of skeletal development; detailed studies of the fetal soft tissues have not yet been done. Comparisons between skeletal development in the control and insulin-treated groups of fetuses are limited to litters counted as having had 22 days of gestation.

Results. Among the rats that delivered their young spontaneously no differences in length of gestation (21 to 23 days) could be discerned between the control and insulin-treated groups. The majority of the rats in both groups were, however, killed on the 22nd day of pregnancy for removal of the fetuses. The average weight of the fetuses with 22-day gestation periods was 5 g in the control group, 4.1 g in the insulin-treated groups.

The control series of 41 rats not treated with insulin yielded 371 fetuses, an average of 9 per litter. The 55 rats treated with insulin yielded 417 fetuses, an average of 7.6 per litter. Counts of resorption sites in the uteri accounted for the differences in litter size. Among 24 rats that received 7 units of insulin per day, 24 resorption sites were counted in 9 (15 showing none). Among 31 rats that received 8 units daily, 87 resorption sites were counted in 24 (only 7 showing none). The total number of 117 resorption sites added to the total number of fetuses in these two groups would bring the number of conceptions to 528, or 9.9 per litter. Among the control rats resorption sites were rarely seen.

On superficial examination all the fetuses from both control and insulin-treated rats appeared normal, with one exception. This one was in a litter of 13, with average weight 3.7 g, which were removed alive from a rat that received 7 units of insulin daily. This fetus was edematous and appeared to have short legs, with poor differentiation of the fingers and toes, and a crooked tail; it was fixed in formalin and serial sections made for histologic studies to be reported later. Among fetuses cleared and stained for skeletal visualization, no noteworthy abnormalities were found in the control group, but a number of abnormalities appeared among fetuses of the insulin-treated group. Photographs in Fig. 1

11. Dawson, A. B., *Stain Technol.*, 1926, v1, 123.

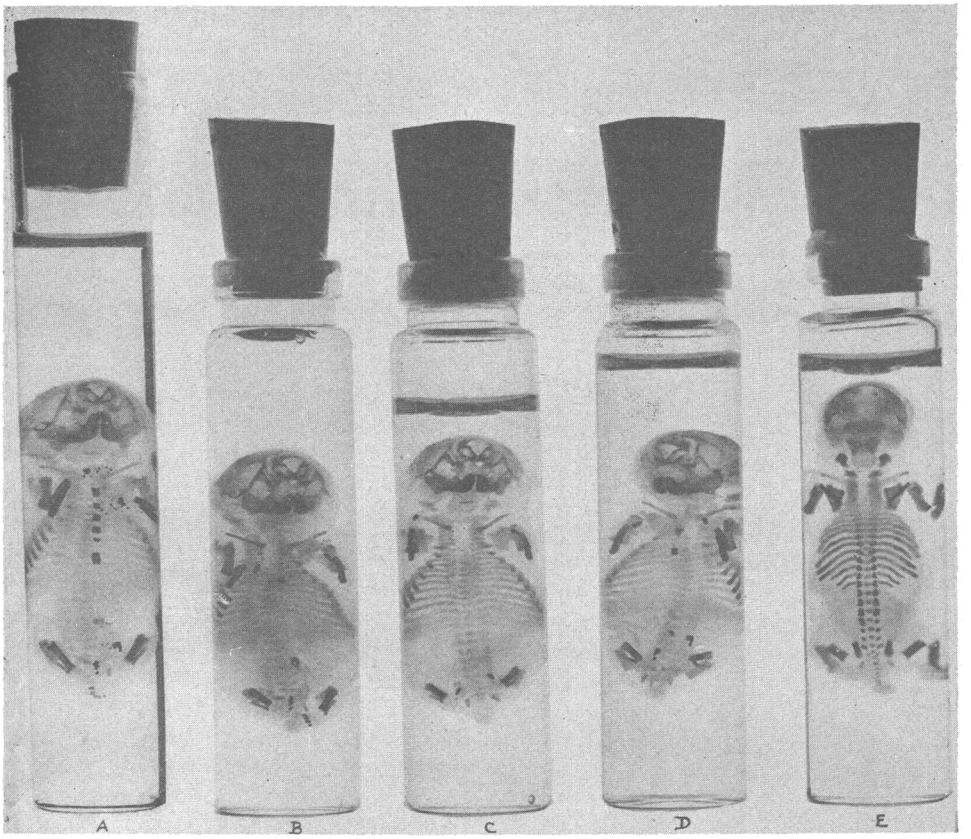

FIG. 1. Fetuses cleared and stained by the Schultze-Dawson method: one (A) from a normal control rat and 4 (B, C, D, E) from rats that received 8 units of protamine zinc insulin daily, all with gestation periods 22 days. A. Normal sternum with 6 well stained sternebrae. B. One well stained sternebra. C. No stained sternebrae. D. Two well stained sternebrae. E. Rear view, showing "wavy" ribs.

and 2 illustrate the absence of visible sternebrae and abnormalities of ossification in the ribs and long bones of the extremities in a few fetuses selected as examples. Among fetuses of rats that received 7 units of insulin daily, "wavy" ribs were seen in 2 fetuses in each of 3 litters, without other visible abnormalities of ossification. Among 4 litters of rats that received 8 units daily, wavy ribs were seen in 1 fetus of each of 2 litters, and in 3 fetuses of each of 2 other litters. In some of the cleared fetuses of the insulin-treated rats the femur and humerus seemed short in relation to body size; occasionally, irregularities were visible in the ends of the femur and tibia, suggesting irregular ossification.

In Table I are listed the numbers of sternebrae seen among cleared and stained fetuses of the control and insulin-treated groups. In the control group of 28 litters with a gestation period of 22 days, 83 cleared fetuses showed 6 well stained sternebrae and 1 showed 5. The latter was in a litter in which the average fetal weight was only 3.6 g. Among 53 cleared fetuses from 11 litters of rats that received 7 units of insulin daily, 50 showed 6 visible sternebrae (some of them poorly stained); of the remaining 3, two showed 3 stained sternebrae and one none. Among 129 cleared fetuses from rats that received 8 units of insulin daily, 66 had 6 sternebrae (visible though many were poorly stained); among the remaining 63 fetuses the visible sternebrae ranged in number from 5 to 0.

Discussion. Strong(12) states that the

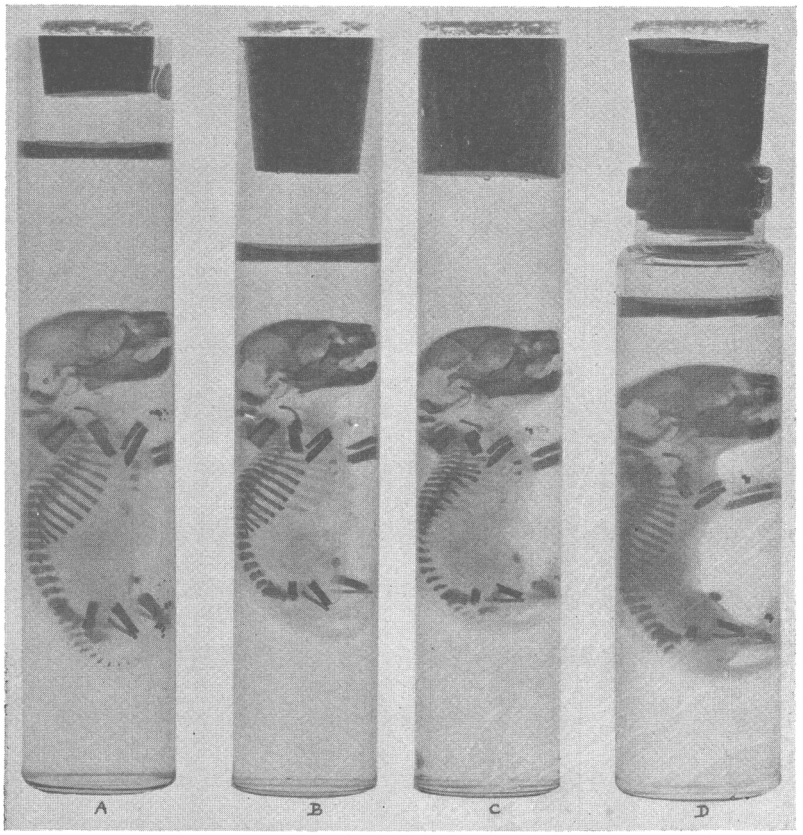

FIG. 2. One fetus from a normal control rat and 3 from rats that received 8 units of protamine zinc insulin daily, all with gestation periods 22 days. A. Normal sternum with 6 well stained sternebrae, normal ribs and normal long bones. B. One well stained and 2 poorly stained sternebrae. C. Three well stained and 3 poorly stained sternebrae, irregular formation of the radius and ulna. The right humerus and the femura are short. The tibiae end obliquely and their staining is irregular. "Wavy" ribs. D. No sternebrae are stained. The femurae are short. The tibia ends are oblique and irregular.

sternum of the albino rat consists of 6 sternebrae. After 19 days, 9¾ hours of gestation ossification centers are already present in all except one or two, and at 21 days all 6 sternebrae are ossified. This was essentially the condition found in our control litters with 22-day gestation period.

In the fetuses from insulin-treated rats the decreased number and/or lack of ossification centers in the sternum, and differences in depth of staining, suggests abnormalities in ossification of the centers, possibly other developmental abnormalities in cellular structure. There appeared to be a parallel comparable retardation of development of the carpals, metacarpals, the phalanges and the vertebrae of the tail.

Since the average weight of fetuses from the insulin-treated rats was less than that of normal controls, it seems possible that the insulin slowed fetal development, directly or indirectly. It is uncertain whether insulin passes through the placenta of the rat(13), and what role changing levels of sugar in maternal and fetal blood may play.

Allen suggested(14) that insulin, especially protamin zinc insulin, may have toxic effects other than those ascribable to hypoglycemia;

12. Strong, R. M., *Am. J. Anat.*, 1926, v36, 313.

13. Schlossman, H., *Arch. Exp. Path. u. Pharmakol.*, 1931, v159, 213; *Chem. Abst.*, 1931, v25, 4052.

14. Allen, F. M., and Vincens, C. A., *Endocrinology*, 1939, v24, 230.

TABLE I. No. of Ossified Sternebrae in Fetuses of Control and Insulin-Treated Rats with 22 Days Gestation.

Stained sternebrae	28 litters —Control— No. of fetuses	%	11 litters —Insulin, 7 units/day— No. of fetuses	%	29 litters —Insulin, 8 units/day— No. of fetuses	%	
6	83	98.8	50	94.3	66	51.16	
5	1	1.2	0	0	10	7.75	66.66
4	0	0	0	0	10	7.75	
3	0	0	2	3.8	13	10.08	
2	0	0	0	0	11	8.53	33.34
1	0	0	0	0	10	7.75	
0	0	0	1	1.9	9	6.98	
Total	84		53		129		

this possibility must of course be considered in the interpretation of results such as those reported here. Duraiswami(9) found, however, that inactivation of insulin by the methyl-alcohol-hydrochloric acid method of Charles and Scott rendered it ineffective in producing abnormalities in developing chicks; in his experiments the effect of insulin on developing chick embryos appeared to be linked with its blood-sugar lowering action. After noting that the skeletal abnormalities induced by insulin in chicks resembled the abnormalities produced in rats by maternal diets deficient in riboflavin, as reported by Warkany and coworkers(1-3), Duraiswami speculated on the possibility that insulin induced hypoglycemia might involve disturbances in phosphorylating enzyme systems such as arise with a lack of vit. B complex, thus causing disturbances of carbohydrate metabolism in embryonic tissue during critical periods of development. Giroud's studies(15,16) on the effects of avitaminosis-B_2 on embryonic development in the rat also are pertinent to such speculations.

Summary. Protamine zinc insulin administered to 51 pregnant albino rats throughout pregnancy in doses 7 and 8 units daily (nearly the maximum dose tolerated) had significant effects on fetal development. Compared with fetuses of untreated normal control rats, the average weight of the fetuses of the insulin-treated rats was lower, and the number in each litter was less owing to fetal deaths and resorption. Mild skeletal abnormalities were found, many of the fetuses showing a decreased number or complete absence of ossification centers in the sternum, a few showing some irregularities in shape and staining of the ribs and long bones.

15. Giroud, A., and Boisselot, *J. Arch. Franc. pédiat.*, 1947, v4, 317.

16. Giroud, A., Lévy, G., et Lefebvres-Boisselot, J., *Revue internationale de Vitaminologie*, 1950, v22, 308.

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Effect of Protein Depletion on Rate of Disappearance of Radioactive Protein from the Blood Stream. (19085)

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The relationship of the effective circulating blood volume and disease states has assumed an important place in surgical physiology. Several methods of determining the blood volume have been advanced, including the use of radioactive (I-131) iodinated plasma