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THE EFFECT OF CHLORTHIAZIDE ON HYPOXANTHINE DEHYDROGENASE, LACTIC DEHYDROGENASE, AND SOLUBLE PROTEIN OF CHICK EMBRYO KIDNEY

S. CHAUBE and C. E. CARTER

Department of Pharmacology, Yale University School of Medicine, New Haven, Connecticut

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Abstract—Chlorthiazide was administered to chick embryos *in vivo* and hypoxanthine dehydrogenase activity, lactic dehydrogenase activity, and the electrophoretic characteristics of the soluble fraction of embryonic renal tissues were subsequently examined. The soluble protein fraction of embryonic renal tissue was altered in embryos which had been treated with chlorthiazide, and this change was associated with a large decrease of hypoxanthine dehydrogenase specific activity.

INHIBITION of avian kidney hypoxanthine dehydrogenase by chlorthiazide *in vitro*[1] indicated that the affinity of the drug for renal enzymic protein might be sufficiently high to produce, *in vivo*, effects upon the developing embryonic chick kidney. In this paper the results of experiments are reported in which chlorthiazide was injected into the chick embryo and the activities of hypoxanthine dehydrogenase and lactic dehydrogenase, and the electrophoretic characteristics of the embryonic renal soluble proteins, were determined. It was found that the renal embryonic tissue was sensitive to the actions of chlorthiazide with a resultant depression of hypoxanthine dehydrogenase activity and an associated change in the electrophoretic pattern of the soluble tissue protein. Under certain conditions, lactic dehydrogenase activity was moderately depressed by chlorthiazide, but changes in the specific activity of this enzyme were relatively small as compared to that observed with hypoxanthine dehydrogenase.

METHODS

Materials

Chlorthiazide was purchased from Merck, Sharp and Dohme Company, diphosphopyridine nucleotide (DPN) and the reduced coenzyme (DPNH) from Pabst Laboratories.

Rhode Island Red eggs were obtained from a local farm and were incubated at 37·8 °C in a constant temperature wooden incubator with humidity adjusted at approximately 60 per cent. To insure optimal growth the eggs were rotated once daily.

All instruments used were placed in 90 per cent alcohol for 1 hr before use.

Embryos

Embryos were classified according to the stages proposed by Hamburger and Hamilton[2] for the normal development of chick. The average incubation time quoted by these authors for each stage was used; thus, the morphological development is expressed as corrected incubation time.

Injection of eggs

After the desired period of incubation, the eggs were candled to reveal the eyeball of the embryo reflected on the shell as a prominent black spot and the allantois as a faintly outlined circular vesicle. The restricted extra-embryonic area between these two structures was used for making the injections into the egg. Through a small aperture in the shell in this area, 0·1 ml of chlorthiazide solution containing the appropriate concentration of the drug in water, was slowly injected into the egg. After injection, the aperture in the shell was swabbed with alcohol, sealed with a piece of scotch tape, and the eggs were transferred to the incubator. During this operation damage to the embryo or yolk sac was carefully avoided and embryos which were accidentally injured were discarded. Control embryos were injected with 0·1 ml of distilled water alone.

Preparation of extracts of embryonic renal tissue

Embryos and membranes were removed from the egg and washed with ice-cold Ringer solution. The embryos were then separated from accessory membranes, weighed and transferred to a dissecting dish. The kidneys, including metanephric and mesonephric tissues, were removed from the surrounding structures and weighed. Approximately 0·5 g of tissue was extracted in a glass homogenizer in 1·5 ml of a 0·05 M solution of sodium phosphate buffer (pH 8·0) at 0 °C and the homogenate was centrifuged for 20 min at 34,000 **g** at 4° C. The supernatant solution was pipetted into 1-ml glass tubes immersed in an ice bath. Enzyme assays were performed on fresh supernatant fractions of homogenates. Embryonic liver extracts were prepared by the same procedure.

Hypoxanthine dehydrogenase

The enzyme activity, which occurred only in the kidney supernatant fraction, was measured by the increase in absorption at 340 mμ resulting from the obligatory reduction of DPN[1] which occurs during the oxidation of hypoxathine. The reaction mixture contained 0·05 μmoles of DPN, 0·05 μmoles of hypoxanthine, 1·0 ml of 0·1 M solution of tris buffer (pH 8·0) and an aliquot of the tissue extract (0·1 or 0·2 ml). Incubations were carried out in 1-ml silica cuvettes at room temperature in the ultraviolet spectrophotometer and absorbancy was determined at 340 mμ, in comparison with a blank which contained the above mixture except for the enzyme solution. The absorbancy of each sample was read at zero time, at 5 min, and then at regular intervals of 10 min, for a total period of 40 min.

Lactic dehydrogenase

Lactic dehydrogenase activity, which was found only in the supernatant fraction, was measured by the decrease in absorption at 340 mμ which results from the enzymic oxidation of the reduced coenzyme, DPNH.[1] The reaction system was composed of 0·5 μmoles of sodium pyruvate, 0·5 μmoles DPNH, 1·0 ml of 0·05 M tris buffer (pH 7·8) and 0·1 ml of the enzyme solution; the final volume was 1·2 ml. The absorbancy of the reaction mixture was compared with that of a blank containing all of the above components except DPNH. Since the activity of this enzyme is higher than that of hypoxanthine dehydrogenase, the supernatant fraction of the renal tissue was diluted (usually 1:80) in order to obtain rates of DPNH disappearance; these were

linear for a 10-min observation period. Activity of the enzyme was then expressed as μmoles of DPNH-decrease/min per mg of soluble protein.

Protein determination

The Folin–Ciocalteau method for determining proteins, as modified by Sutherland *et al.*,[3] was employed.

Starch block electrophoresis was carried out on extracts of embryonic kidney by the method described by Paigen,[4] and Bloemendal.[5]

RESULTS

Fig. 1 depicts the effects of several concentrations of chlorthiazide, injected into the embryo on the fifth day of incubation, upon hypoxanthine dehydrogenase activity of

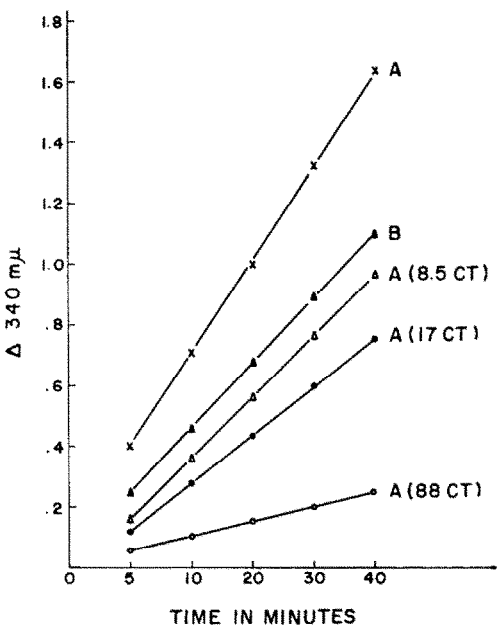

FIG. 1. Hypoxanthine dehydrogenase activity, estimated as increment of optical density at 340 mμ (DPNH) over a 40-min incubation period with supernatant fractions of embryonic kidney. Embryos were injected with three dosages (88, 17 and 8·5 μmoles) of chlorthiazide (CT) per egg on the fifth and harvested on the eighth day of incubation. Kidney supernatant fraction aliquots (A, 0·2 ml; B, 0·1 ml), prepared from 0·3 g of tissue from untreated 8-day embryos, were compared with 0·2-ml aliquots of the supernatant fraction prepared from an equivalent weight of embryonic renal tissue of chlorthiazide-treated eggs (A, CT, 8·5; A, CT, 17; and A, CT, 88).

kidneys removed on the eighth day of incubation. These data show that the formation of DPNH in the enzymatic reaction increased linearly over the 40-min period of the assay, and that enzyme activity was proportional to the amount of the supernatant fraction added. In these experiments, chlorthiazide produced over 50 per cent inhibition of renal hypoxanthine dehydrogenase activity when 17 μmoles of the compound were injected per egg.

In the chicken embryo, renal excretory function is carried out principally by the mesonephric kidney until the twelfth to fifteenth day when the metanephric kidney becomes functional.[6] The two renal organs are separable and since both function in

the 15-day embryo, the effect of chlorthiazide on hypoxanthine dehydrogenase in these tissues was examined. A comparison of the activities of hypoxanthine dehydrogenase and lactic dehydrogenase was also made, as shown in Table 1. Preliminary experiments indicated that in the 15-day-old egg 43 μmoles of chlorthiazide produced from 60 to 70 per cent inhibition of hypoxanthine dehydrogenase and this was compatible with the survival of over 90 per cent of the embryos; these conditions also

TABLE 1. THE EFFECT OF CHLORTHIAZIDE ON HYPOXANTHINE DEHYDROGENASE AND LACTIC DEHYDROGENASE OF RENAL TISSUE FROM 15-DAY EMBRYOS*

	Hypoxanthine dehydrogenase specific activity		Lactic dehydrogenase specific activity	
	Mesonephros	Metanephros	Mesonephros	Metanephros
Control†	21×10^{-3}	17×10^{-3}	2·0	1·4
CT-Day 5‡	3×10^{-3}	11×10^{-3}	1·2	1·5
CT-Day 14§	7×10^{-3}	6×10^{-3}	1·9	1·1

* Fourteen embryos were employed in each experimental group. Mesonephric and metanephric tissues were dissected from the embryo and 0·3 g mesonephric tissue and 0·9 g metanephric from each group were separately pooled and extracted with 1·0 ml of 0·1 M Tris buffer, pH. 7·8. The preparation of the supernatant fraction and definition of specific activity are described in the Methods section.

† Separate groups of control embryos were injected with 0·1 ml of distilled water on day 5 and on day 14 and both groups were harvested on day 15. Since differences in the hypoxanthine and Lactic dehydrogenases between the two groups was in the range of $\pm$1·2–2, averages were obtained and are shown in the table.

‡ 43 μmoles of chlorthiazide in 0·1 ml of distilled water were injected on day 5 and the embryos were harvested on day 15.

§ 43 μmoles of chlorthiazide in 0·1 ml of saline were injected on day 14 and the embryos were harvested on day 15.

were achieved when 17 μmoles of chlorthiazide were injected into the 8-day egg. The data of Table 1 show that chlorthiazide administration on the fifth day of incubation produced a depression of hypoxanthine dehydrogenase activity, when this was determined on the fifteenth day in both the mesonephric and metanephric renal tissue, but that the magnitude of the inhibitory effect was greatest in the mesonephros. The activity of lactic dehydrogenase, assayed under these conditions, was decreased in the mesonephros to a lesser extent than was that of hypoxanthine dehydrogenase. Lactic dehydrogenase activity was not depressed in the metanepheric kidney. These results demonstrate that chlorthiazide can produce long-term effects upon the developing embryonic kidney, and that these effects are most pronounced in the tissue which was performing the excretory function at the time of the injection of the drug. When chlorthiazide was injected into the embryos on day 14 and the enzyme activities were assayed on day 15, lactic dehydrogenase was found to be relatively insensitive to the action of the drug. Hypoxanthine dehydrogenase activity was depressed over 60 per cent in both metanephric and mesonephric tissues under these conditions. In none of the cases of chlorthiazide-induced depression of hypoxanthine dehydrogenase activity *in vivo* was there evidence of reversibility of the inhibition upon addition of excess substrate. *In vitro*, the inhibition was found to be reversible.[1]

The specific activity of the enzymes reported in Table 1 was based upon the amount of soluble protein of the supernatant fractions of kidney tissues. From these data, it

appeared that injection of the drug might be capable of altering the soluble protein component of the developing embryonic kidney. Such an effect was demonstrated in the diagrams (Fig. 2) obtained from starch block electrophoresis[4, 5] of embryonic renal tissues under experimental conditions similar to those described in Table 1. Electrophoresis of supernatant fractions was conducted on extracts prepared from the entire embryonic kidney, which included mesonephric and metanephric tissue, because of the requirement of this technique for larger amounts of starting material. The protein diagram (Fig. 2) of the supernatant fractions, prepared from equivalent

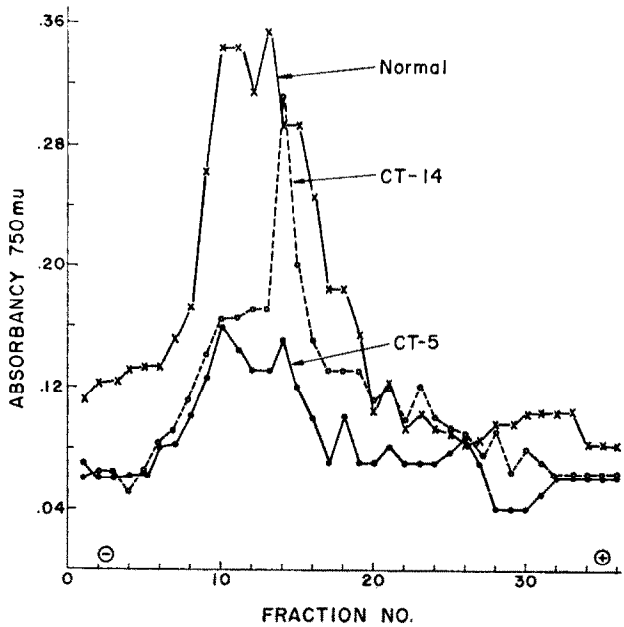

FIG. 2. Electrophoresis of the soluble proteins of the supernatant fraction of control and chlorthiazide treated 15-day embryonic kidneys. Metanephric and mesonephric tissues were combined and in each experimental series 1·2 g of tissue derived from fourteen to sixteen embryos were homogenized in 2·0 ml of 0·1 M Tris buffer, pH 7·8. The amount and time of injection of chlorthiazide were the same as described in Table 1. The starch block electrophoresis was conducted on 1 ml of the supernatant fraction for 22 hr at 400 V in the apparatus described by Paigen,[4] as modified by Bloemendal,[5] using 36-cm troughs. The buffer system employed was 0·1 M Tris, pH 7·8. From the starch blocks, 1-cm sections were cut and eluted in distilled water, 2·0 ml, 4 °C for 24 hr. Eluates were examined spectrophotometrically for absorbancy at 260 mμ (nucleic acid), and 0·2-ml aliquots of a 1 : 10 dilution of the eluate were employed for protein determination. The amount of protein in the fractions is expressed as 750 mμ-absorbancy of the colored product of the Folin reaction.[3] The diagram designated CT-14 represents the distribution of acid-soluble protein from the renal supernatant fraction of embryos which had received 43 μmoles of chlorthiazide per egg on day 14 and were harvested on day 15. The designation CT-5 represents the results of an experiment in which 43 μmoles of chlorthiazide were administered on day 5 and the embryos were harvested on day 15.

weights of control and chlorthiazide-treated embryos, demonstrated alterations attributable to the action of the drug. Embryos injected on the fifth day of incubation with 43 μmoles of chlorthiazide and harvested on the fifteenth day for preparation of renal extracts, showed an extensive and uniform depression of the principal components of the electrophoretic diagram. Embryos injected on the fourteenth day with

chlorthiazide (43 μmoles) and harvested on the fifteenth day showed a great decrease in the 9-cm component and a relatively smaller depression of the 14-cm component. Since the same weight of embryonic kidney was used in each preparation, it is apparent that the administration of chlorthiazide was associated with changes in the soluble protein components of embryonic chick renal tissue. Since both hypoxanthine dehydrogenase and lactic dehydrogenase exhibited peak specific activity in the 9-cm fraction, the electrophoretic data offer no explanation for the selective depression of the hypoxanthine enzyme (Table 1). However, the relatively low activity of hypoxanthine dehydrogenase, as compared with that of dehydrogenase in the normal tissue, may make changes in the ratio of enzyme protein to total protein, as measured by catalytic activity, disproportionately large.

The pattern of nucleic acid distribution determined by the absorption at 260 mμ of the electrophoretically separated extract, revealed a single peak with maximum absorption in the 11-cm fraction. Samples from the drug-treated tissues which exhibited the change in protein distribution shown in Fig. 2, showed no alteration in the nucleic acid distribution and no decrease in the amount of nucleic acid, as evidenced by the absorption at 260 μm.

DISCUSSION

Administration of chlorthiazide to chick embryos *in vivo* produced alterations in the proteins of the supernatant fraction of renal extracts. A decrease in hypoxanthine dehydrogenase activity was an indication of the change, but was probably a relatively non-specific consequence of the action of the drug. Under appropriate conditions a lesser effect was observed on lactic dehydrogenase, an enzyme which exhibits high activity in the renal supernatant fractions. The mechanism of the effects of chlorthiazide on the soluble proteins of embryonic chick renal tissue is not known. However, the data presented in this paper demonstrate the sensitivity of the developing chick kidney to the effects of this drug and the suitability of the tissue for investigation of the effects of chlorthiazide on the synthesis of renal soluble protein. Whether effects of a similar nature could be obtained in mammalian tissues remains to be determined, but certain observations indicate that analogous behavior would not be anticipated. First, renal hypoxanthine dehydrogenase, an enzyme obligatorily linked to DPN, does not occur in mammalian tissues.[1] Second, the observation that the extensive use of chlorthiazide in pregnancy in human subjects has not been associated with complications of fetal life, would tend to indicate that the developing chick kidney is uniquely sensitive to the actions of the drug. This sensitivity may be attributed to the metabolic characteristics of the chick kidney, or to the "closed" system of fluid cf compartments in the egg which permit attainment of high concentrations of chlorthiazide at embryonic renal sites, a circumstance not encountered in the mammalian embryo because of the placental and maternal circulation.

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