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Effect of Chlorothiazide on Rat Liver Glucokinase, Hexokinase and Dihydroxyacetone-Kinase Activities.* (30011)

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Salas, Vinuela and Sols(1) and Sharma, Manjeshwar and Weinhouse(2) reported on a glucose phosphorylating enzyme, glucokinase, the synthesis of which, in contrast to hexokinase, is insulin dependent. This enzyme is virtually absent in the liver of alloxan diabetic rats, but with repeated insulin injections its normal level can be restored in 50 to 100 hours. The activity of another enzyme, dihydroxyacetone-kinase, which phosphorylates dihydroxyacetone (DHA), also has been shown to be insulin dependent(3,4). This enzyme, too, is absent from the liver of the alloxan diabetic rat. With injection of insulin to the diabetic animal, normal enzyme levels can be restored in 20 to 30 hours. A decrease in plasma insulin-like activity has been reported after administration of benzothiadiazine drugs(5). The following studies were carried out to determine if chlorothiazide administration to rats alters liver glucokinase, hexokinase and DHA-kinase activities.

Methods. Eighteen non-fasting male Sprague-Dawley rats weighing 250 to 350 g were given 100 mg chlorothiazide† per kg body weight per day in drinking water for 14 to 18 days. Eighteen similar control animals drank tap water. Animals were sacrificed by decapitation. Glucokinase and hexokinase activities were determined by the method of Salas, Vinuela and Sols(1). Livers were perfused *in situ* with 30 ml of a solution containing 150 mM KCl, 5 mM sodium EDTA, and 5 mM $MgCl_2$ at pH 7.0. They were then removed, weighed and homogenized in a glass-Teflon homogenizer with 2 volumes of cold buffer containing 100 mM Tris-HCl, 1 mM sodium EDTA and 1 mM β-mercaptoethanol at pH 7.4. Homogenates were then centrifuged at 2°C for 30 minutes at 108,000 *g*. Supernatant solutions were assayed for glucokinase and hexokinase activities at room temperature (22-23°C). These assay systems, designated C, contained in 3 ml final volume 0.2 i.u. glucose 6-phosphate dehydrogenase, 0.25 mM NADP, 5 mM ATP, 5 mM $MgCl_2$, 5 mM β-mercaptoethanol, 50 mM Tris-HCl at pH 7.4, 0.1 M glucose and 0.05 ml freshly prepared supernatant solution. Four additional assay systems were carried out which differed from C as follows: B had 0.5 mM instead of 0.1 M glucose, A had 50 mM N-acetyl-glucosamine instead of 0.1 M glucose, D had 0.25 mg chlorothiazide added per ml, and E had 1 unit of glucagon-free insulin‡ added. C-B gives glucokinase activity and B-A gives hexokinase activity(2). Rates of NADPH formation were divided by 1.7 to correct for the contribution of endogenous 6-phosphogluconate dehydrogenase(2).

For DHA-kinase activity determinations, 22 similar rats were given 100 mg chlorothiazide per kg body weight per day in drinking water for 14 to 18 days and 25 control animals drank tap water. Rats were sacrificed by decapitation and their livers re-

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TABLE I. Effect of Chlorothiazide Administration on Glucokinase, Hexokinase and Dihydroxyacetone-Kinase Activities of Rat Livers.

Enzymes	Chlorothiazide rats	Normal rats
Glucokinase*	.80 ± .16 (18)	1.31 ± .15 (18)
Hexokinase*	.20 ± .03 (18)	.18 ± .02 (18)
DHA-kinase†	.75 ± .28 (22)	1.22 ± .21 (25)

* Expressed as mean ± S.D. of μM glucose phosphorylated per min per g liver(1).

† Expressed as mean ± S.D. of units of DHA-kinase per mg protein when 1 unit equals 0.1 μM DHA phosphorylated per hr at 37°C(4).

Values in parentheses represent No. of livers assayed. Each assay was done in duplicate.

moved, immediately weighed and rinsed in a cold buffer solution at pH 7.4 containing 0.01 M sodium phosphate and 0.001 M sodium EDTA. The tissue was homogenized in 1.5 times its weight of buffer in a glass-Teflon homogenizer. The soluble fraction was obtained by centrifuging the homogenate at 195,000 *g* for one hour at 2°C and collecting the clear supernatant solution.

The disappearance rate of DHA was determined(3). A cold incubation mixture containing 0.05 ml 0.03 M NAD, 0.05 ml 0.02 M ATP, 5 μl 0.154 M $MgSO_4$, 0.65 ml of tissue homogenate supernatant solution and 0.25 ml 0.01 M sodium phosphate buffer at pH 6.9, was warmed to 37°C. At zero time 50 μl 0.2 M DHA were added, immediately mixed and a sample was promptly removed and its protein precipitated. Additional samples were removed at 10 minutes. DHA contents were measured by the method of Tsao and Schwartz(6). Metaphosphoric acid (0.2 M) was used for precipitation of protein instead of barium hydroxide and zinc sulfate. The protein contents of the homogenates were estimated by the biuret reagent.

Results. Values for glucokinase, hexokinase and DHA-kinase activities of liver homogenates of chlorothiazide treated and normal rats are shown in Table I. Rats given chlorothiazide had a 40% diminution in liver glucokinase activity and a 30% reduction in liver DHA-kinase activity. Both of these values are significantly different from those of normal rats ($p<0.001$). Hexokinase activity of the liver homogenates was slightly increased by chlorothiazide administration to the rats. Addition of chlorothiazide to the assay mixture did not change the rate of glucose phosphorylation by glucokinase as determined in 17 normal rats (1.35 ± 0.17 μM) or in 17 chlorothiazide rats (0.83 ± 0.20 μM). Similarly addition of chlorothiazide to the medium did not change the rate of phosphorylation by DHA-kinase as determined in 20 normal rats (1.20 ± 0.25 units) or in 12 chlorothiazide rats (0.72 ± 0.30 units).

Discussion. These experiments demonstrate that administration of chlorothiazide to rats results in a decrease in liver glucokinase and DHA-kinase activities. As the synthesis of these enzymes is dependent on insulin, these lowered enzyme levels might reflect reduced insulin-like activity of plasma resulting from chlorothiazide administration. This is supported by the finding that hexokinase, which is not insulin-dependent, is not decreased by giving chlorothiazide to rats. Addition of chlorothiazide to the assay system did not change the rate of phosphorylation of glucose or DHA, showing that this drug is not acting as a direct inhibitor of glucokinase or DHA-kinase. It is possible that chlorothiazide or its metabolic products might decrease the amounts of glucokinase and DHA-kinase enzymes formed through a mechanism other than by decreasing plasma insulin-like activity. The evidence, however, strongly suggests that chlorothiazide administration causes decreased production of both glucokinase and DHA-kinase by diminishing the level of plasma insulin-like activity.

Summary. Administration of chlorothiazide to rats decreases the glucokinase and DHA-kinase activities of their liver homogenates but does not diminish liver hexokinase activity. Addition of chlorothiazide directly to the assay system has no effect on glucokinase or DHA-kinase activity.

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Species Specific Blood Groups in *Cynopithecus niger* (Celebes Ape).* (30012)

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The creation of primate research centers throughout the country has led to an increased interest in blood groups of primates, particularly as they relate to man. Aside from study of human blood groups, much of the early work was concerned with study of the antigenic structure of erythrocytes of Old World monkeys, particularly the species *Macaca mulatta* (rhesus monkey). This work has been reviewed recently by Wiener(1). Investigations were directed toward revealing factors related to human blood group factors, and it was through these investigations that the Rh_0 (D) factor of human red cells was discovered. More recently, investigations have centered on the extent of human blood group factors in anthropoid apes. Relatively few studies have been concerned with intra-species differences among lower primates with the exception of *M. mulatta*(2,3). This report will present data revealing species-specific blood factors present on red cells of the adult Celebes ape *(Cynopithecus niger)*.

A systematic survey of blood for species-specific blood groups in the lower primates using rabbit immune sera, after appropriate absorptions, was undertaken at the Oregon Regional Primate Research Center in 1962 (4). Attention was focused on the reactions of anti-rhesus erythrocyte serums absorbed with blood cells of the homologous species or of the Celebes ape. Suggestive intra-group differences among rhesus monkeys and among Celebes apes were noted, particularly in the latter, but these differences were not marked and the results were not easily reproducible. After a period of inactivity in this area of investigation, we undertook to reevaluate and expand the work previously done in this laboratory.

Materials and methods. Antisera were prepared by injecting rabbits with red cells from 5 selected apes; each rabbit was injected with cells from a single donor. Two series of injections were given. The first series consisted of 2 injections of 0.5 ml of a 50% suspension of cells given 5 days apart. The second series was given 5 weeks later and consisted of an intraperitoneal injection of 0.5 ml followed by an intravenous injection 2 days later. Four weeks later the animals were given a booster dose by intraperitoneal injection and were bled out 5 days later. Serums were separated from clotted cells, inactivated for 30 minutes at 56°C and stored in the freezer after addition of sodium azide to a final concentration of 0.1%.

Titrations of all serums were performed using human red cells of various ABO types, rhesus red cells and Celebes ape red cells. In addition, 2 serums were titrated using erythrocytes from 2 baboons (Papio species), 1 pig-tailed macaque *(Macaca nemestrina)*, 1 Sykes monkey *(Cercopithecus mitis)*, 3 tree shrews *(Tupaia glis)*, and 6 animals from 2 species of lemurs (*Lemur catta* and *Lemur fulvus*). Titrations were performed making 2-fold dilutions in saline in a volume of 0.05 ml and adding 1 drop of 2% suspension of test cells. Saline agglutinations were read after incubating for 15 minutes at room temperature followed by centrifugation for 30 seconds at 3500 rpm. One drop of bromelin

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