Effect of Age upon Collagen and Hexosamine Content of Rat Skin.* (23820)

J. C. Houck and R. A. Jacob
Surgical Research Metabolic Laboratory, Georgetown University Hospital, Washington, D.C.

Sobel and his co-workers have found that the hexosamine and collagen content of rat skin increased with rat body weight(1). They have also shown that, since the collagen concentration increased more rapidly than the hexosamine concentration, the ratio of hexosamine to collagen decreases with age(2). We have studied these components in the skin of animals weighing more than 250 g, the upper weight limit used by Sobel *et al.* The results of the analyses for the hexosamine, hydroxyproline and nitrogen contents of rat skin are reported below.

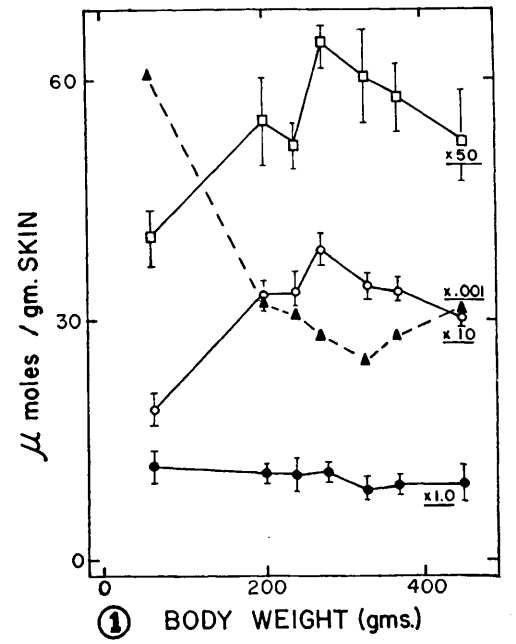
Materials and methods. Preparation of tissue. Wistar strain female rats ranging in weight from 50 to 500 g were grouped into 7 weight classifications, with 6 rats in each group. These animals were fed on Fox Chow pellets *ad lib.* After killing by etherization, a section of dorsal skin of each rat was shaved, and about 2 square cm of tissue removed. This tissue was carefully dissected clean of adhering fat and muscle, and stored in frozen state. After thawing, tissues were weighed, minced and autoclaved with 10 ml/g of 4 N HCl for 3 hours. The samples were then incubated at 100°C for 2 hours, at which time duplicate 0.1 ml samples were removed for nitrogen and hydroxyproline determination. After 5 hours incubation, 1 ml samples were removed for hexosamine analysis. These heating times were optimal for hydrolysis to hexosamine and hydroxyproline, while quantitative recoveries of these compounds could be affected from the tissue mixture. The results of these analyses were expressed in μmoles/g of fresh, wet, tissue. *Analysis for hexosamine.* The 1 ml samples were neutralized with 4 N NaOH and diluted with 2 ml of acetyl acetone-carbonate reagent(3) and 1 ml of 4 N NaCl. This mixture was treated according to Blix(3). The results were translated *via* a standard curve into μmoles g fresh tissue. *Analysis for nitrogen.* The appropriate 0.1 sample was diluted 300 fold with water and 0.1 ml of this solution was mixed with 0.2 ml of a digestion mixture of 50% sulfuric acid containing 1% SeO_2. After digestion this mixture was diluted to 7 ml with water and 3 ml of Koch and McMeekin Nessler solution was added. The resulting optical density at 430 mμ was translated *via* a standard curve into μmoles/g of fresh tissue. *Analysis for hydroxyproline.* 0.1 ml samples were diluted to 2.5 ml with a 4 N HCl. 0.5 ml of this solution was diluted to 5 ml with water and 1 ml samples were then analysed for hydroxyproline by the method of Martin and Axelrod(4).

Results. The mean weight of each group of rats was 65, 200, 240, 280, 330 and 450 g. The correlation between body weight, and hence the age of rat skin, and mean concentration of hydroxyproline, hexosamine and nitrogen/g of tissue and standard deviation of each group is shown in Fig. 1. Although the amount of hydroxyproline increased with age in animals weighing up to 280 g, there was a statistically significant decrease in concentration of this component with animals of greater weight. There was essentially no change in amount of hexosamine/g of fresh rat skin over the whole range of animal weights studied. The change in total nitrogen of whole tissue closely paralleled the hydroxyproline determination. The ratio of hexosamine to hydroxyproline concentration declined in animals weighing from 65 to 330 g. Above this weight however, the ratio increased significantly.

Since variation in water content of connective tissue occurs with age, it was decided to relate hexosamine and hydroxyproline content of rat skin to total nitrogen of tissue. The results are illustrated in Fig. 2. The ratio of hexosamine to hydroxyproline was naturally unchanged. The ratio of hydroxyproline to nitrogen was changed only in that the maximum occurred in animals weighing 240 g. The shape of curve illustrated in Fig. 2 was

* Supported in part by grant from Nat. Inst. of Arthritis and Metabolic Diseases, P.H.S.

relatively unchanged from that presented in Fig. 1. The effect of animal weight upon hexosamine to nitrogen ratio as shown in Fig. 2, was profoundly altered from that described in Fig. 1. Concentration of hexosamine relative to that of nitrogen decreased with body weight up to 330 g. Above this weight size, however, the ratio increased with further increases in animal body weight. These changes were closely paralleled by the effect of body weight upon hexosamine to hydroxyproline ratio.



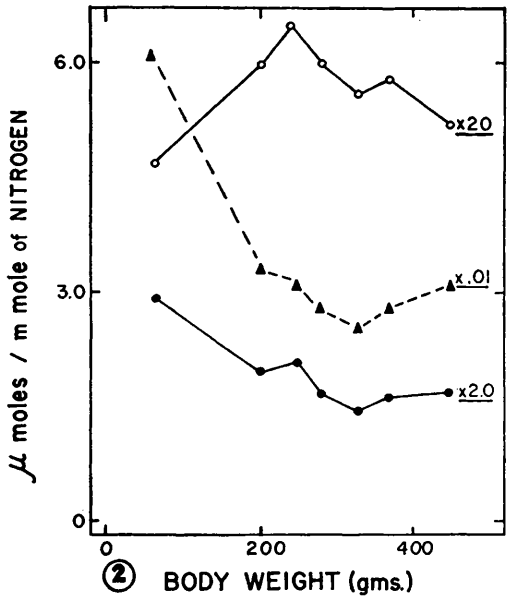


FIG. 1 (top). Effect of body wt upon concentration of hexosamine (●), hydroxyproline (○) and nitrogen (□) in rat skin, and upon ratio of hexosamine to hydroxyproline (▲). Values expressed per g of fresh whole skin.

FIG. 2 (bottom). Effect of body wt upon ratio of hexosamine (●) and of hydroxyproline (○) to total tissue nitrogen, and upon ratio of hexosamine to hydroxyproline values expressed per μmole of nitrogen (▲).

Discussion. Contrary to data presented by Sobel and his co-workers(1,2) in their study of rats weighing to 250 g, we have been unable to find any significant change in hexosamine content of skin, unless the results are expressed relative to total nitrogen of tissues. If the data are so presented then the concentration of hexosamine does not increase but rather decreases in the skin of rats up to about one year in age. With animals older than this (body weight greater than 330 g) the relative concentration of hexosamine increases. The close correlation between effect of age upon relative hexosamine content of rat skin and the ratio of hexosamine to hydroxyproline indicates that formation of mucopolysaccharides may be a critical feature of the ageing process. The parallel response of hydroxyproline and tissue nitrogen to age suggests that there is no disproportionate alteration in skin proteins with increasing body weight.

The presentation of hexosamine and hydroxyproline data relative to the total nitrogen of tissue has some obvious advantages; a) the tedious fat and water extraction procedures are not required, b) concentrations of the components are expressed relative to total protein of tissue, and c) a third component, extraneous protein, is thereby indirectly measured.

Summary and conclusions. 1) Concentration of hexosamine in whole rat skin is relatively unaffected by animal age, although the amount of this material relative to total tissue protein is progressively decreased with increasing body weight up to 330 g. Both the total and relative concentrations of hydroxyproline in rat skin are increased with animal weight up to 280 or 240 g respectively. Above this weight class the amount of hydroxypro-

line decreased slightly. Total tissue nitrogen parallels these changes. 2) Above 330 g however, the relative hexosamine concentration increases with body weight. The effect of body weight upon the ratio of hexosamine to hydroxyproline closely parallels the response of relative concentration of hexosamine to rat age.

1. Sobel, H., Zutrauen, H., Marmorsten, J., *Arch. Biochem. and Biophys.*, 1953, v46, 221.
2. Sobel, H., Marmorsten, J., *J. Gerontol.*, 1956, v11, 2.
3. Blix, G., *Acta. Chem. Scand.*, 1948, v2, 467.
4. Martin, C. J., Axelrod, A., PROC. SOC. EXP. BIOL. AND MED., 1953, v83, 461.

Received December 26, 1957. P.S.E.B.M., 1958, v97.

Seizure Patterns in Young Animals. Significance of Brain Carbonic Anhydrase. II.*† (23821)

J. GORDON MILLICHAP (Introduced by Henry L. Barnett)
Departments of Pediatrics and Pharmacology, Albert Einstein College of Medicine, N. Y. City

Previous work has demonstrated a direct relation between the activity of brain carbonic anhydrase and the susceptibility of animals to experimental seizures. In mice, acetazolamide has been shown to be approximately twice as potent as sulfanilamide as an inhibitor of brain carbonic anhydrase and as an anticonvulsant; the maximum degree of inhibition of brain enzyme corresponded with the time of peak anticonvulsant effect of each drug(1). In addition, it has been shown recently in newborn animals of different species that the development of susceptibility to various experimental seizure patterns is directly related to the level of carbonic anhydrase in the brain(2). In rats between 20 and 35 days of age, in which a high level of brain carbonic anhydrase was observed, it was possible to induce both major and minor seizure patterns by variation of the strength of an electroshock stimulus. A current of 50 m. amps. applied for 0.2 sec. was the threshold stimulus required for induction of the maximal seizure pattern, which consists principally of tonic extension of the hind-limbs (grade 5); tonic flexion or extension of the forelimbs with clonus (grade 4) was induced by a stimulus of 40 m. amps.; and symmetrical clonic seizures with loss of posture (grade 3) occurred in response to a threshold current of 30 m. amps. Stimuli of 20 and 10 m. amps. respectively were required for the induction of catatonic (grade 2) and hyperkinetic (grade 1) minor seizure patterns. In rats younger than 20 days, the grade of seizure pattern obtained in response to an electroshock stimulus of 50 m. amps. was less severe than that observed in older rats between 20 and 35 days of age, and the level of brain carbonic anhydrase was correspondingly lower. In the newborn, a hyperkinetic grade 1 seizure response was associated with a minimal amount of carbonic anhydrase in the brain. With a rapid increase in enzyme activity between 10 and 20 days, seizure patterns of grades 2, 3 and 4 were obtained.

In the present investigation, designed to test further this apparent relation between carbonic anhydrase activity in the brain and various patterns of seizure activity, the anticonvulsant effect of acetazolamide has been observed in parallel with estimations of the degree of inhibition of the enzyme.

Methods. Effect of acetazolamide on seizure patterns. The effect of acetazolamide on seizure patterns induced by electroshock was determined in albino rats (Holtzman strain) between 28 and 32 days of age and weighing between 75 and 100 g. Acetazolamide sodium was dissolved in distilled water and administered by subcutaneous injection. Since tolerance develops on repeated injection(3,4), in-

* This investigation was supported in part by grant from Nat. Inst. of Neurological Diseases, N.I.H.
† Acetazolamide (Diamox) was supplied by Lederle Medical Research Department, Pearl River, N. Y.