Chemical Dissection of Rat Skin.* (26098)

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The primary anatomical components of connective tissue are fibers, cells and ground substance(1). Hydroxyproline is a quantitative measure of the major fiber, collagen (2), while hexosamine is a measure of ground substance mucopolysaccharides and glycoproteins(3). Uronic acid is largely confined to the acid mucopolysaccharides(4). Proteins and, to a lesser extent, urea, nitrogenous bases, hexosamine and amino acids contribute to total dermal nitrogen content.

This paper describes both the analysis of cleaned, shaven rat skin with respect to these materials and water and fat, and the extractive dissection of rat skin into ground substance, neutral soluble collagen(5), procollagen(6) and insoluble collagen.

Material and methods. Twelve male Sprague-Dawley rats averaging 330 g in weight were sacrificed by etherization and exsanguination, and after shaving about 5.5 g of abdominal skin were removed and dissected clean of adhering fat, fascia and muscle. After mincing, the tissue was either lyophilized or stored in the frozen state. Subsequently, portions of these tissues were hydrolysed at 100°C for 8 hr in 10 ml/g of 4N HCl. The acid hydrolysates were then analysed for hexosamine(7) uronic acid(8) and hydroxyproline(9). Nitrogen was determined by digesting in triplicate 0.10 ml of a 1:10 dilution of acid hydrolysate with 0.2 ml of 50% H_2SO_4 containing 1% SeO_2 at 270°C in a Halikanen digestion box for 2 hours, and Nesslerization(10). These conditions for hydrolysis and digestion were optimal, and quantitative recoveries of added collagen, mucopolysaccharide, hydroxyproline or hexosamine could be effected thereby(11).

The remainder of the tissue samples were extracted for 45 minutes at 4°C with 10 ml/g of 0.15 M NaCl at 17,500 rpm in a Lourdes Tissue Blender and the homogenate allowed to stand over night in the cold. After centrifugation at 25,000 × g for 1 hr. the clear supernatant was removed, and the residue re-extracted similarly with 0.50 M NaCl. The residue from this second extraction was in turn extracted with 20 ml/g of 0.50 M citrate buffer (pH 3.6) under similar conditions.

Repeated extraction of rat skin with each solvent resulted in the further release of less than 15% of the hydroxyproline or hexosamine found in the first extract under the conditions described above.

The hydroxyproline, hexosamine, nitrogen and uronic acid content of these extracts was determined after HCl hydrolysis as described above.

Results and discussion. Theoretically, most of the free mucopolysaccharides and glycoproteins of the ground substance, and contaminating serum glycoproteins, would be soluble in isotonic saline. The residue of this extraction contained morphologically intact cells and collagen, but no longer stained profoundly with Alcian blue, a histochemical reagent for acid mucopolysaccharides(12). The chemistry of this extract of rat skin has been exhaustively studied by Sweeney(13) and Mathieson(14).

The extrafibrillar precursor of collagen is soluble in 0.5 M NaCl(5). The residue from this extraction contained collagen fibers which, under the electron microscope, had lost a portion of their surface material and appeared somewhat "leached." The cells had been distorted and destroyed.

Citrate soluble procollagen from rat skin has been studied in detail by Orekovitch and Shpikiter(6). The residue after extraction by this solvent contained grossly swollen, distorted collagen bundles and gluey masses of fibers. Subtraction of the sum of the various soluble components from the appropriate total concentration found in the whole skin permits determination of the insoluble dermal constituents to be made. The residue of the sequential extraction procedure described

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TABLE I. Extractive Dissection of Normal Rat Skin.

Extract	Hydroxyproline, μmoles/g	Hexosamine, μmoles/g	Nitrogen, mmoles/g
.15M NaCl	7.7 ± 3	5.1 ±.3	1.30 ±.06
.50M "	29 ± 2	2.4 ±.1	1.20 ±.06
.50M citrate buffer	12 ± .6	.10	.60 ±.04
Insoluble	312 ±13	4.4 ±.2	3.90 ±.2
Total	360 ±15	12.0 ±.6	7.00 ±.3

above was so swollen (9×) and non-homogeneous that direct measurements were singularly unreliable. The results are presented in Table I.

Because of the large amount of color contributed to the carbazole method by dermal hexoses, uronic acid determinations were unreliable. Quantitatively, however, most of the uronic acid was found in the isotonic saline extract, and very little in the other soluble fractions. Table I then is concerned solely with the hexosamine, hydroxyproline and nitrogen content of the various fractions. More than 42% of the total dermal hexosamine was soluble in isotonic saline, while only a trace of this amino sugar appeared in the citrate fraction. About 37% of the hexosamine was completely insoluble and presumably represents either a glycoprotein or a mucopolysaccharide bound to the collagen fibers.

Purified collagen contains about 18.6% nitrogen or 13.3 μmoles/mg and 13.6% hydroxyproline or approximately 1.1 μmole/mg (15). Ratio of hydroxyproline to nitrogen (μmole/mmole) for highly purified collagen is about 83. A similar ratio for the insoluble dermal material is about 80, therefore this component is almost pure collagen. The extra nitrogen contaminating this fraction probably results from keratin and elastin. The purity of this collagen precludes the insoluble hexosamine being glycoprotein, since this material would contain almost as much nitrogen as simple protein. Therefore insoluble collagen is intimately associated with mucopolysaccharide. This is consistent with the claim of Stoianova *et al.*(16) that collagen fibrils are surrounded by a sheath of mucopolysaccharide which is removed by hyaluronidase activity.

The non-protein nitrogen of normal skin was studied by measuring nitrogen content of the trichloracetic acid supernatant of the saline fractions. Five percent of the nitrogen in the isotonic saline extract and 3% of the 0.5 M saline soluble nitrogen was not precipitated by this treatment.

The amount of dermal nitrogen accounted for by collagen may be determined by multiplying the hydroxyproline content of the tissue, since 1 μmole equals 1 mg of collagen, by 13.3. Dermal collagen accounts for about 70% of total nitrogen. The difference between total nitrogen and collagen nitrogen would be due largely to non-collagenous protein and glycoprotein.

By lyophilization, it was found that fresh, cleaned, shaven skin contained about 550 mg/g H_2O, and after standing in the frozen state for 5 days, all skin samples contained 250 mg/g. Further storage for 25 more days did not appreciably decrease this value(11). The data of Table I was obtained using stored skin, and could be corrected with respect to water loss *via* sublimation during storage in the frozen state by multiplication by 0.68(11). These corrected values agreed within 10% with those obtained using fresh tissue. Accordingly, fresh rat skin contained 240 mg of collagen, 150 mg of other protein and 550 mg of water per g. The difference between 1000 and the sum of these 3 dermal constituents must be accounted for by fat, or 60 mg/g. Kao *et al.*(17) have reported total fat content of the skin of 8-month-old male rats (approx. 230 g) to be 74 mg/g.

Summary and conclusions. Extraction of rat skin with 0.15 M NaCl in the cold removes almost half of the dermal hexosamine and most of the uronic acid, leaving a residue which no longer stains profoundly with Alcian blue, although cells and collagen of the tissue are intact. Further extraction with 0.50 M NaCl removes about 8% of total dermal hydroxyproline leaving a residue in which neither the cells nor the collagen are intact. The citrate buffer soluble hydroxyproline (3% of the total) is not contaminated

with hexosamine, while ⅓ of total dermal amino sugar is found intimately associated with the insoluble residue, probably as a mucopolysaccharide. This residue is highly purified dermal collagen. Finally, fresh skin contains 55% water, 6% fat, 24% collagen and 15% non-collagen protein and glycoprotein.

1. Florey, H. W., *Lectures on General Pathology*, Phila., W. B. Saunders Co., 1954, p. 21.

2. Neuman, R. E., Logan, M. A., *J. Biol. Chem.*, 1950, v186, 549.

3. Boas, N. F., *Biochem. Biophys. Acta*, 1955, v57, 367.

4. Meyer, K., Rapport, M. M., *Science*, 1951, v113, 596.

5. Gross, J., Highberger, J. H., Schmitt, F. O., *Proc. Nat. Acad. Sci.* (Wash.), 1955, v41, 1.

6. Orekovitch, V. N., Shpikiter, V. O., *Science*, 1958, v127, 1371.

7. Blix, G., *Acta. Chem. Scand.*, 1948, v2, 467.

8. Dische, Z., *J. Biol. Chem.*, 1950, v183, 489.

9. Martin, C. J., Axelrod, A. E., PROC. SOC. EXP. BIOL. AND MED., 1953, v84, 461.

10. King, E. J., *Microanalyses in Medical Biochemistry*, J. and A. Churchill, Ltd., London, 1950, p. 12.

11. Houck, J. C., Jacob, R. A., Maengwyn-Davies, G. D., *J. Clin. Invest.*, 1960, Nov., in press.

12. McManus, J. F. A., *Histochemistry of Connective Tissue* in Connective Tissue in Health and Disease. Ed., Asboe-Hanses, Munksgaard, Copenhagen, 1954, p. 35.

13. Sweeney, P., *Chemical Analysis of Rat Skin*. Ph.D. thesis, Univ. of Western Ontario Med. School Library, London, Ontario, 1959.

14. Mathieson, J., *Chemical Analysis of the Isotonic Saline Extract of Rat Skin*. M.Sc. thesis, *ibid.*, 1959.

15. Gustavson, K. H. *The Chemistry and Reactivity of Collagen*. New York Academic Press, 1956, pp. 37-50.

16. Stoianova, I. G., Nekrasova, T. A., Zaides, A. L., *Dokladii Akad. Nauk SSSR*, 1960, v130, 1366.

17. Kao, K. Y. T., Schwartz, J., Treadwell, C., McGavack, T., PROC. SOC. EXP. BIOL. AND MED., 1960, v103, 666.

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Metabolism of Cortisol by the Liver of Sodium Deficient Rats.‡ (26099)

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A major step in the metabolism of corticosteroids is reduction of the Δ^4-3-ketone group(1). It has been shown that rat liver contains steroid Ring A hydrogenases capable of catalyzing this reduction(2). Urquhart *et al.*(3) and Yates *et al.*(4) have proposed that rate of hepatic reduction of adrenal cortical steroids is a controlling factor in rate of secretion of those steroids by the adrenal through a negative-feedback mechanism by which the anterior pituitary (ACTH) regulates plasma corticosteroid concentration. It is likely then that conditions which produce changes in adrenocortical secretion rates might also alter the ability of the liver to metabolize active corticosteroids.

Eisenstein and Hartroft(5) have shown that total steroid secretion by the isolated adrenal of sodium deficient rats was significantly less than that of controls. The purpose of this study was to evaluate the effect of sodium deprivation on capacity of rat liver to reduce the Δ^4-3-ketone group of cortisol.*

Methods and materials. Female white rats of the Carworth strain (maintained on Purina pellet chow), weighing 140-200 g, were divided into two groups. One group (21 rats) was fed a commercially† prepared synthetic low-sodium diet which included all the nutrients essential for normal growth except sodium and contained 14.6 mg Na^+/100 g of diet and 910 mg K^+/100 g of diet. The other group (20 rats) was fed a diet† similar in all respects to the low-sodium diet except that

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* Δ^4-pregnene-11β,17α,21-triol-3,20-dione.

† Nutritional Biochemicals Corp.