of the vinyl ether double bond. The experiment involving tritiated water was designed to test the possibility of hydrogenation involving one or more hydrogen ions from water. When compared to a glucose-6-C^{14} control sample, no increased incorporation of tritium into glyceryl ethers due to tritium uptake by the side chain was noted. However, since the over-all amount of radioactivity incorporated was small and since it was not determined into which position or positions of the glyceryl moiety tritium was incorporated, the validity of these results must be confirmed by further experimentation. Catalytic hydrogenation experiments of the type reported here promise to be more useful in studying the role of plasmalogens as possible glyceryl ether precursors.

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The Biochemistry of Plasmalogens. III. Concentrations in Tissues of the Rat as a Function of Age*

EUGENE L. GOTTFRIED AND MAURICE M. RAPPORT†
WITH THE TECHNICAL ASSISTANCE OF MRS. PHYLLIS ROSEN

From the Departments of Biochemistry and Medicine and the Unit for Research in Aging, Albert Einstein College of Medicine, Yeshiva University, New York

Received March 7, 1963

The plasmalogen concentrations of heart, lung, liver, spleen, and skeletal muscle were determined by two independent methods in rats 1, 2, 3, 4, and 8 weeks of age. In contrast to the marked increase noted in brain during this period of rapid growth, no consistent change in plasmalogen concentration with age was observed in any of these tissues when comparisons were made on the basis of fresh weight of tissue, total lipid content, or total phosphatide content. A simple ultramicro spectrophotometric method for specific iodination has been found to be satisfactory for determination of the plasmalogen content of total lipid extracts.

It was established by Minder and Abelin (1954) and confirmed by Korey and Orchen (1959), Erickson and Lands (1959), and Bieth *et al.* (1961) that the aldehydogenic phosphatide content of the brain of the rat increases severalfold as the animal increases in age. In contrast, it was found (Minder and Abelin, 1954) that in other organs (heart, kidney, muscle, and liver) the plasmalogen content decreases with age, a sharp drop occurring between 3 and 4 weeks. These changes were determined in relation to fresh weight of tissue. Only three groups of animals were compared, namely, 3-week-old, 4-week-old, and "adult" rats. We have restudied this problem in order to develop a more complete picture with respect both to age and to other bases of reference, e.g., total lipid and total phosphatide. Five organs (heart, lung, liver, spleen, and muscle) were studied in five groups of rats (1, 2, 3, 4, and 8 weeks old). A more reliable method than that available to Minder and Abelin (Schiff reaction) was used to determine the aldehydogenic phosphatide content, namely, *p*-nitrophenylhydrazone formation (Wittenberg *et al.*, 1956). Plasmalogens were also measured by the more specific determination of α,β-unsaturated ethers (Rapport and Franzl, 1957). The findings of Minder and Abelin regarding change with age in heart, muscle, and liver could not be confirmed.

* This work was supported by research grants B-1570, A-2965, H-3838, and M-2562, National Institutes of Health, U. S. Public Health Service.

† American Cancer Society Professor of Biochemistry.

Experimental Procedure

Sprague-Dawley albino rats (Holtzman strain) used in this study were kept with their mothers, with free access to a stock Rockland rat diet. Groups of five rats each were sacrificed at ages 1, 2, 3, 4, and 8 weeks. Animals in the first group were sacrificed by decapitation; for the others, ether anesthesia was used, followed by immediate dissection. The selected organs were trimmed, washed in 0.9% saline solution, blotted quickly on a paper towel, and weighed. The specimens were then stored in ice until processed (within 2 hours). Pools of tissues were ground in a glass homogenizer or a Waring Blendor with 20 volumes of chloroform-methanol (2:1, v/v) at room temperature. The chloroform-methanol extracts were then filtered and washed by the method of Folch *et al.* (1951). Lipids

were stored in solution in chloroform-methanol (2:1) at −10° until analyzed.

The organs studied were heart, lung, liver, spleen, and skeletal muscle. Muscle specimens consisted of tissue from the left thigh region; the other organs were extracted *in toto*. Methods for determining phosphorus and long-chain aldehydes (by *p*-nitrophenylhydrazone formation) have been described previously (Gottfried and Rapport, 1962).

The α,β-unsaturated ether content of these tissues was measured by a spectrophotometric iodination method (Gottfried and Rapport, 1962), adapted for the analysis of crude lipid extracts. Lipid samples containing 0.01 to 0.09 μmole of α,β-unsaturated ether were treated as previously described. In addition, two blank values were determined: (1) I_2 reagent, no sample; (2) 3% KI instead of I_2 reagent, plus sample. The reference cuvet contained 3% potassium iodide–methanol–ethanol (1:1:8). Iodine uptake was determined as follows: ΔOD (corrected) = OD (I_2) + OD (KI + sample) − OD (I_2 + sample). Then, I_2 uptake (μmoles) = $\frac{\Delta OD \text{ (corrected)}}{5.50}$ [molar absorbancy index (a_m or ϵ), 27,500].

Thin-layer chromatograms were made of all extracts, using plates prepared with silica gel G (Merck) and a developing solvent of chloroform-methanol-water (75:25:4) at room temperature, as previously described (Gottfried and Rapport, 1963). Spots were detected by exposure to iodine vapors and by spraying with Schiff reagent (Gottfried and Rapport, 1963).

Results

Thin-layer chromatograms of the lipid extracts indicated the presence of major phosphatides with the mobilities of phosphatidyl ethanolamine, phosphatidyl choline, and sphingomyelin (R_F values of 0.60–0.65, 0.35–0.40, and 0.22–0.29 respectively). By means of the Schiff reagent, plasmalogens were found to be present primarily in the ethanolamine phosphatide fraction, although heart had a considerable amount in the choline glycerophosphatide ("lecithin") fraction as well. With liver, the Schiff reagent produced a nonspecific reddish color with both the ethanolamine and the choline glycerophosphatide fractions (in contrast to the deep purple color characteristic of plasmalogens and free aldehydes). In a given tissue, no shift was detected in the phosphatide pattern as a function of age.

The results of chemical analyses of the individual extracts are shown in Figure 1. When plasmalogen concentrations are compared in terms of micromoles of *p*-nitrophenylhydrazone per gram of fresh tissue (Fig. 1*A*), only small differences among the various age groups are seen in all tissues except lung. In the latter, there is an increase over the period from 1 to 3 weeks (from 2.0 to 3.6 μmoles), followed by a decline (to 2.5 μmoles) by the 8th week. These changes largely reflect the degree of hydration of the fresh lung tissue, for the plasmalogen content per unit weight either of total lipid (Fig. 1*B*) or of total phosphatide (Fig. 1*C*) changed only to a small extent. With heart tissue, the ratio of plasmalogen content to total lipid weight declined with age (Fig. 1*B*); this change was not seen in comparisons based on either phosphatide content (Fig. 1*C*) or fresh weight (Fig. 1*A*). Skeletal muscle plasmalogen showed a small decline from 1 to 8 weeks with reference either to fresh weight or to total phosphatide, but not with reference to total lipid weight.

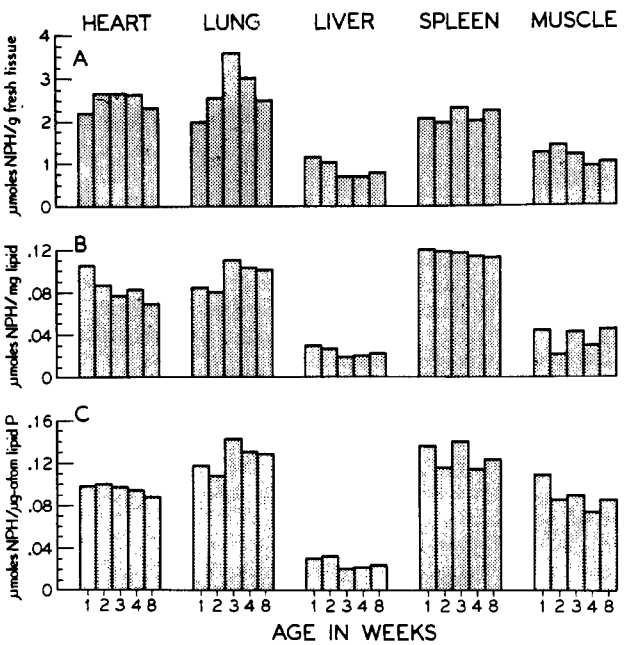

FIG. 1.—Plasmalogen content of rat tissues as a function of age. Aldehydogenic lipids are measured as the *p*-nitrophenylhydrazones (NPH): (*A*) μmoles per gram of fresh tissue; (*B*) μmoles per mg lipid; (*C*) μmoles per μg-atom of lipid phosphorus.

Comparison of the α,β-unsaturated ether content (by spectrophotometric estimation of specific iodination) with aldehydogenic lipid content (by *p*-nitrophenylhydrazone formation) indicated close correspondence, except in the case of liver. The average molar ratios of α,β-unsaturated ether to aldehydogenic lipid (I_2/NPH) were as follows: heart, 0.96; lung, 1.09; liver, 0.65; spleen, 1.09; and skeletal muscle, 1.10.

Discussion

The study described here was designed to confirm and extend the report by Minder and Abelin that the plasmalogen content of rat heart, kidney, muscle, and liver decreased with age, and might thus be related to function. Three of the four tissues cited in their report (heart, muscle, and liver) were included and two were added (lung and spleen). The ages 1, 2, 3, 4, and 8 weeks were substituted for theirs of 3 weeks, 4 weeks, and the indeterminate "adult." In addition to the reference base "fresh weight of tissue" used by Minder and Abelin, we have also included total lipid weight and lipid phosphorus.

The results of this study do not confirm the findings of Minder and Abelin. The plasmalogen concentrations in non-nervous tissues such as heart, lung, liver, spleen, and skeletal muscle do not, indeed, show the marked increase during the first 8 weeks seen in the brain (and attributable to myelination), but neither do they appear to decrease significantly. The phospholipid composition of these tissues during this period of rapid growth does not appear to undergo any basic change.

The discrepancy between our results and those of Minder and Abelin may in part be attributed to their use of a less reliable analytical method (Schiff reaction); it is more likely to be the result of some variable other than age. In addition to biological variation, factors such as exercise, reported to decrease the plasmalogen content of skeletal muscle (Thiele *et al.*, 1961), must be considered. Diet may be of considerable importance. It is possible that the sharp drop in plasmalogen con-

tent observed by Minder and Abelin in comparing the 3- and 4-week-old animals may be related to the abrupt dietary change that takes place with weaning (usually at about 3 weeks of age). In contrast, such an abrupt change was avoided in the experiment reported here by allowing the rats to remain with their mothers.

If, as present evidence suggests, plasmalogens are specifically localized in cell membranes, then the relatively constant phospholipid pattern implies that there is no further differentiation in terms of formation of membrane material (cellular or subcellular) in such tissues as heart, lung, liver, and spleen during the period from 1 to 8 weeks.

The spectrophotometric iodination method for measuring α,β-unsaturated ethers, originally developed to characterize relatively pure phosphatide mixtures (Gottfried and Rapport, 1962), has been found to be quite useful for analysis of crude lipids when only very small amounts of sample are available. For example, in the pooled spleens of 1-week-old rats, the entire sample contained only 0.8 μmole of α,β-unsaturated ether. Values for the molar ratio of I_2/NPH obtained with this method with extracts of various rat tissues other than liver agree well with those previously reported by Rapport and Lerner (1959) and Norton (1960). In the case of rat liver lipids, the average value found in this study was 0.65, which is close to that recorded by Norton (0.71) and much lower than values reported by Rapport and Lerner (0.90). This discrepancy has recently been found by Camejo *et al.* (1963) to result from interference by vitamin A in crude extracts.

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Evidence that Steroid Sulfates Serve as Biosynthetic Intermediates: *In vivo* Conversion of Pregnenolone-Sulfate-S^{35} to Dehydroisoandrosterone Sulfate-S^{35}*

Harold I. Calvin,† Raymond L. VandeWiele,‡ and Seymour Lieberman

From the Departments of Biochemistry and Obstetrics and Gynecology, College of Physicians and Surgeons, Columbia University, New York

Received January 21, 1963

Pregnenolone sulfate-S^{35} (NH_4^+ salt) (1.1×10^7 cpm) was administered to a female subject with adrenal cancer who was excreting 300 mg/day of 17-ketosteroids. A tracer amount of tritiated dehydroisoandrosterone sulfate (1.7×10^5 cpm) was added during the processing of the first 24-hour urine collection, and the endogenously produced dehydroisoandrosterone sulfate was isolated by celite partition chromatography. The conjugate was purified by successive recrystallization to a constant H^3/S^{35} ratio (4.9), which was not altered by conversion to the oxime of dehydroisoandrosterone sulfate. Thirteen per cent of the administered S^{35} was extracted by the Edwards-Kellie-Wade procedure from the first day's urine (1.4×10^6 cpm), and of this 2.5% (3.5×10^4 cpm) was recovered as dehydroisoandrosterone sulfate-S^{35}. That pregnenolone sulfate may serve as a direct *in vivo* precursor of dehydroisoandrosterone sulfate reveals a heretofore unsuspected and still undefined metabolic role for sulfate conjugates.

The classical view of steroid hormone metabolism has assumed that the hormones are synthesized as free steroids in endocrine tissue, and then prepared for excretion in urine by peripheral metabolism and conjugation. Recently, however, evidence has been obtained that dehydroisoandrosterone,[1] the chief precursor of the 17-ketosteroids found in urine (Vande-

* Supported in part by USPHS Grant A 110 and Naval Research Grant NONR 266(75). A preliminary report of this work has appeared (Calvin *et al.*, 1963).

† Predoctoral Research Fellow, National Institutes of Health.

‡ Investigator of the Health Research Council of the City of New York.

[1] The following trivial names and abbreviations have been used throughout the text: dehydroisoandrosterone = 3β-hydroxy-5-androsten-17-one; dehydroisoandrosterone sulfate = 3β-sulfoxy-5-androsten-17-one; androsterone = 3α-hydroxyandrostan-17-one; etiocholanolone = 3α-hydroxyetiocholan-17-one; androstenedione = 4-androstene-3,17-dione; testosterone = 17β-hydroxy-4-androsten-3-one; pregnenolone = 3β-hydroxy-5-pregnen-20-one; pregnenolone sulfate = 3β-sulfoxy-5-pregnen-20-one; 17α-hydroxypregnenolone sulfate = 3β-sulfoxy-17α-hydroxy-5-pregnen-20-one; pregnenediol-3-monosulfate = 3β-sulfoxy-5-pregnen-20α-ol.