utilizing alkaline hydrolysis could not be used with this material because of the high peptide contamination of the RNA fraction resulting from treatment with alkali. However, the perchloric acid method was considered to be satisfactory, since DNA contamination of the RNA fraction was negligible (less than 2 percent), and there was no evidence for substantial losses of RNA into the DNA fraction.

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Adrenal Cholesterol: Localization by Electron-Microscope Autoradiography

Abstract. *As determined by electron-microscope autoradiography of adrenal glands containing tritiated cholesterol and by modified differential centrifugation techniques, 70 to 80 percent of adrenal cholesterol is contained within lipid droplets of rat adrenal cortical cells.*

Five percent of the wet weight of the adrenal cortex of the rat is comprised of cholesterol, most of which is esterified. This large pool of cholesterol is depleted after adrenocorticotropic hormone (ACTH) stimulation of adrenal steroidogenesis (*1*); cholesterol is a direct precursor of adrenal steroid hormones (*2*). Although adrenal cholesterol has not been definitely localized, mitochondrial fractions prepared by differential centrifugation contain considerable quantities of cholesterol (*3*). Histochemical studies have shown a correlation between the presence of adrenal cholesterol and the cell layers producing corticosterone (*4*) and a depletion of sudanophilic material in the adrenal after administration of ACTH (*5*). Lipid droplets are also depleted after administration of ACTH (*6*). These histologic results suggest that adrenal cholesterol is located, at least in part, in the lipid droplets. The light-microscopic methods for localization of tissue cholesterol lack the specificity or resolution needed to determine subcellular localization (*7*), and biochemical studies on isolated adrenal lipid droplets have not been reported. Because there is evidence that the rate-limiting reaction in production of adrenal steroid hormones is the transformation of cholesterol to pregnenolone and that the enzyme system responsible for the reaction is present within the mitochondrion (*8*), determination of the intracellular location of the substrate (cholesterol) is important.

Sprague-Dawley rats (200 g) were injected intravenously with 500 μc of H^3-cholesterol previously conjugated to rat serum protein (*9*). Eighteen hours later, the specific activities of adrenal and plasma cholesterol were within 90 percent of equilibrium, and maximum incorporation of radioactivity into adrenal cholesterol was obtained (*10*). Chromatography of adrenal lipids revealed that more than 99.5 percent of the radioactivity contained in the adrenal was present as cholesterol (*11*). Adrenals were fixed with 1 percent glutaraldehyde in modified Tyrode's solution (*12*) perfused through the abdominal aorta. The adrenals were cut into small blocks and rinsed in phosphate-buffered 7.5 percent sucrose (*p*H 7.2). Osmium fixation was essential for the retention of cholesterol. When osmium fixation was omitted entirely, almost all the cholesterol was extracted by the dehydrating fluids (Table 1); less than 1 percent remained in the embedded tissue (*14*). With a standard 1-hour fixation in 1 percent osmium tetroxide, 34 percent of the cholesterol was retained in the embedded tissue. These findings are similar to studies by Korn (*15*) which indicate that loss of lipid-soluble substances occurs during preparation of tissue for electron-microscopic studies.

Because osmium may react with lipids by forming complexes with unsaturated lipids (*16*) and because highly unsaturated fatty acids occur in adrenal cholesterol esters (*17*), we treated adrenal tissue with 2 percent osmium tetroxide in a phosphate or barbital buffer at room temperature for 12 to 24 hours. Prolonged fixation with osmium doubled the retention of cholesterol after standard processing for electron microscopy (Table 1). Retention was increased to more than 90 percent by omitting absolute ethanol and propylene oxide and using instead Epon 812 as the final dehydrating agent after 95 percent ethanol [a modification of the procedure of Idelman (*18*)]. This was the best method tried and was used to prepare adrenal tissue for autoradiography. For the latter procedure, lightly carbonized 90-nm sections of Epon-

Table 1. Extraction of H^3-cholesterol from adrenal glands during processing for electron microscopy. Percentage of disintegrations per minute (dpm) retained (second column) is expressed as percent of total dpm (dpm extracted plus dpm retained). The "embedding mixture" was composed of Epon 812, acid anhydrides, and amine (*13*). Specimens described in the bottom row were partially dehydrated up to 95 percent ethanol, were then soaked in Epon 812 alone (three transfers), and finally were infiltrated with the complete embedding mixture.

Fixation after glutaraldehyde	Disintegrations per minute (% dpm)							
	Retained	Extracted						
		Aqueous solutions	Ethanol			Propylene oxide	Epon 812	Embedding mixture
			70%	95%	Abs.			
None	0.6 ± 1.0*	0.1	7.6	36.1	54.1	1.4		0.1
1 percent OsO_4 for 1 hr	32.0 ± 2.9	0.1	0.6	10.4	28.4	28.0		0.5
2 percent OsO_4 for 24 hr	67.2 ± 4.9	0.2	0.5	1.9	7.8	25.7		1.2
2 percent OsO_4 for 24 hr	90.3 ± 2.1	0.2	0.5	1.8			5.7	1.5

* ± 1.0 S.E.M.

Table 2. Relative distribution of cholesterol in rat adrenal glands as determined by differential centrifugation and by electron-microscope autoradiography. A correction for background was applied to grain counts used for calculating figures in the third column.

Cell fraction	Cell fractions prepared by differential centrifugation		Grains over cell organelles (%)
	Total cholesterol by gas-liquid chromatography (%)	H^3-cholesterol (% dpm)	
Lipid droplets	75	80	77
Nuclei	7	5	0.5
Mitochondria	10	3.5	9
Microsomes	8	10	
			13.5*
Soluble	0.1	1.5	

* Adrenal cortical cell cytoplasm, excluding lipid droplets and mitochondria.

embedded tissue from the inner half of the zona fasciculata were coated with Ilford L-4 Nuclear Research Emulsion. The autoradiographs were developed 3 months later in Kodak D-19 or Microdol-X developer.

The developed silver grains from adrenal H^3-cholesterol were located, for the most part, in the lipid droplets (Fig. 1). In order to quantitate this observation, grain counts were tabulated (Table 2) and related to the cross-sectional areas (*19*); a total of 7000 μ^2 was analyzed. Background over the parts of the section devoid of tissue was found to be 2.5 grains per 100 μ^2. The number of grains over nuclei was 3.3 grains per 100 μ^2 (uncorrected for background). Lipid droplets had 95 grains per 100 μ^2, whereas mitochondria had 9, and the remainder of the cytoplasm, 13.7 grains per 100 μ^2 (all uncorrected). After correction for background was made, the specific radioactivity of the lipid droplets was ten times greater than that of the rest of the adrenal cortical cell cytoplasm.

The subcellular distribution of cholesterol in rat adrenal cortical cells was also studied with cell-fractionation techniques. Adrenals from six rats injected with H^3-cholesterol as described above were decapsulated and then were homogenized in 5 ml of a 17 m*M* phosphate buffer (*p*H 7.2) made 153 m*M* in NaCl and 1.0 m*M* in $MgCl_2$ (*20*). The homogenate was centrifuged for 20 minutes at 35,000 rev/min in an SW-39 rotor in a Beckman model L ultracentrifuge. The lipid droplets formed a compact layer at the top of the liquid. Electron-microscopic examination of this layer revealed that it was composed almost entirely of the intact lipid droplets. The remaining cell fractions were prepared by standard techniques (*3*). After purification (*11*), choleseterol from the lipid droplets and from the other cell fractions was quantitated by gas-liquid chromatography. The gas chromatograph was equipped with a 4-foot (1.2-m) column of 3.8 percent SE-30. The carrier gas was helium, the carrier temperature 250°C, and the flame detector was kept at 300°C. A pair of rat adrenal glands contained 696 ± 20 μg of cholesterol, 75 percent of which was found in the lipid droplet fraction (Table 2). Eighty percent of the total radioactivity was found in the lipid droplets (Table 2). The correlation between the localization and distribution of adrenal cholesterol as determined by autoradiography and by differential centrifugation suggests that the cholesterol did not diffuse significantly during processing for electron microscopy.

Because the lipid droplet layer prepared by centrifugation broke up easily and diffused rapidly, considerable care was necessary to prevent contamination of other cell fractions with lipid droplets. Contamination with lipid droplets may account for the varied intracellular distribution of adrenal cholesterol obtained in other studies (*3*).

Our studies indicate that most of the adrenal cholesterol is in the lipid droplets of the adrenal cortical cells. The enzyme system responsible for the conversion of cholesterol to pregnenolone, the rate-limiting step in adrenal steroidogenesis, is in the mitochondria (*8*). Since cholesterol, the substrate for this reaction, is segregated predominantly in the lipid droplets, cellular mechanisms must exist which cause mobilization of the cholesterol from the lipid droplets to the mitochondria when steroidogenesis is stimulated by ACTH.

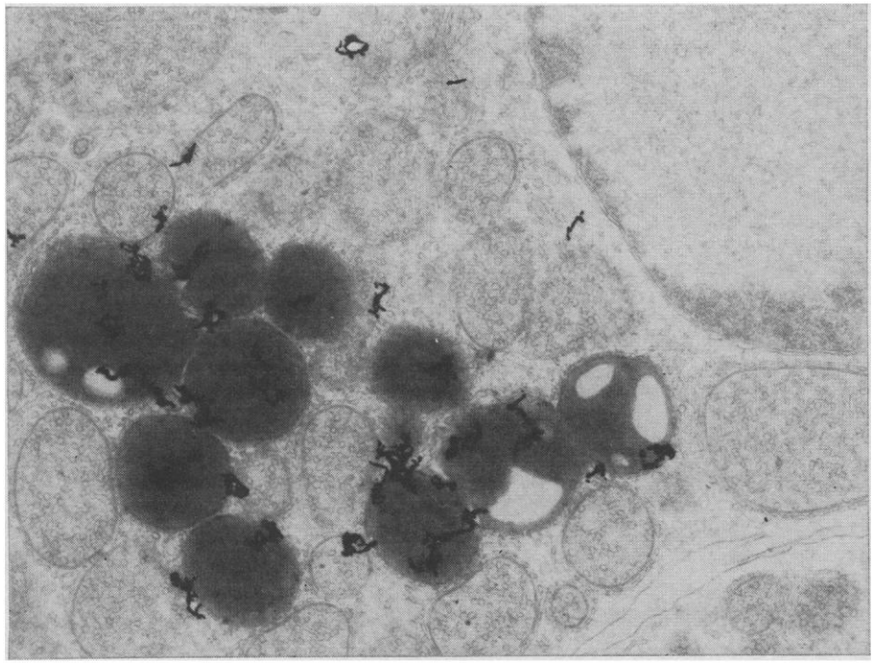

Fig. 1. Electron-microscopic autoradiograph of H^3-cholesterol in rat adrenal cortical cell. The majority of the developed grains are present over the lipid droplets. Developed after exposure for 3 months with Kodak D-19 developer. Stained with aqueous uranyl acetate (× 16,500).

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Nitrification: Importance to Nutrient Losses from a Cutover Forested Ecosystem

Abstract. *The nitrate concentration (weighted average) in stream water from an experimentally deforested watershed increased from 0.9 milligram per liter before removal of the vegetation to 53 milligrams per liter 2 years later. This nitrate mobilization, presumably due to increased microbial nitrification, was equivalent to all of the other net cationic increases and anionic decreases observed in the drainage water.*

Loss of nutrients in drainage water was greatly accelerated after the northern hardwoods vegetation was clear-cut in November and December 1965 on watershed 2 (15.6 hectares) of the Hubbard Brook Experimental Forest in central New Hampshire (*1*). Regrowth of vegetation has been retarded by the application of two herbicides, Bromacil ($C_2H_{13}BrN_2O_2$) and an ester of 2,4,5-trichlorophenoxyacetic acid.

We have measured interactions between the hydrologic cycle and nutrient cycle in several undisturbed watershed ecosystems of the Hubbard Brook Experimental Forest since 1963 (*2*). These watersheds are characterized by a similar vegetation and geologic substrate. The latter forms a watertight basement, which makes it possible to determine quantitative nutrient budgets for these ecosystems from measurements of chemical input and output (*3*).

The chemical concentration in drainage waters from the cutover watershed did not increase significantly until June 1966, 5 months after the forest was cut (*1*). Therefore, milliequivalent values for the water year, 1 June 1965 to 31 May 1966, are equivalent to those for 1964–65 (Table 1), and to those obtained from undisturbed watersheds (*3*). Cations and anions in the drainage water greatly increased during 1966–67 and 1967–68 (*1*) (Table 1). Average concentrations (in milligrams per liter) in stream water increased from 0.9 for NO_3^-, 1.8 for Ca^{++}, 0.4 for Mg^{++}, 0.9 for Na^+, and 0.2 for K^+ in 1965–66 to 38, 6.5, 1.4, 1.5, and 1.9, respectively, during 1966–67, and 53, 7.6, 1.5, 1.5, and 3.0, respectively, during 1967–68. However, the weighted concentration of SO_4^{--} in drainage water decreased by about 45 percent. Because of the increased average concentration (Table 1) and increased streamflow from the cutover watershed, the net budgetary losses during 1966–67 for Ca^{++}, Mg^{++}, Na^+, K^+, and nitrate nitrogen were 9, 8, 3, 20, and 100 times greater, respectively, than those in comparable, undisturbed watersheds during the same period. The magnitude of these losses was great in relation to a comparative lysimeter study in a small cutover plot in a Douglas fir plantation in Washington (*4*). These changes may be accounted for by an increase in microbial nitrification.

Many microorganisms convert organic nitrogen into ammonia. The ammonia then may be oxidized to nitrite by bacteria of the genus *Nitrosomonas*. Nitrite may be further oxidized to nitrate by bacteria of the genus *Nitrobacter*. Our data on nutrient output from the undisturbed forest provide nothing definitive on the occurrence of nitrification (*1*). Others (*5*, *6*) have questioned the occurrence of significant amounts of nitrification in climax forest ecosystems. At any rate, in the absence of vegetation, the microflora of the cleared watershed oxidizes ammonia to nitrate rapidly (*1*). The important end products of these reactions—in terms of nutrient losses from the ecosystem—are the increased amounts of nitrate and hydrogen ions. With decreasing *p*H, cations such as Ca^{++}, Mg^{++}, Na^+, and K^+ are more quickly dissolved and leached from the system. This may occur (i) as the hydrogen ions replace cations on the humic-clay ion exchange complexes of the soil, or (ii) as organic or inorganic compounds are decomposed. Since nitrification occurs in the humic layers of the soil, these base exchanges probably occur concurrently with the decay of organic substances.

In this regard, the ionic composition of the drainage water from the cutover watershed suggests that the predominant source for the surplus cations is the breakdown of humic substances from the upper layers of the soil. This is best shown by the changes in the ratio of Ca to Na in the drainage water of watershed 2 before and after deforestation. Before cutting, the ratio of Ca to Na in the stream water was 1.6:1.0, which is consistent with that observed in adjacent undisturbed watersheds, and has been attributed to the steady-state chemical weathering of bedrock and till (*7*). However, after clear-cutting, the ratio climbed to an average of 4.8:1.0 for 1966–68. More significantly, the ratio for the excess ions of the stream (that is, the amount added above those present in the undisturbed condition) is 7.4:1.0. These ratios show that the net chemical effect of the deforestation was to differentially produce