SIMULTANEOUS USE OF H^3 AND C^{14} COMPOUNDS TO STUDY CHOLESTEROL METABOLISM

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I WOULD like to discuss our experiences using carbon-14 and tritium compounds simultaneously to study intermediary metabolism. While the concurrent use of two different isotopes to study a metabolic reaction is not unique, the wider acceptance of this method as a general technique has been hindered by the technical difficulties involved in the separation and analysis of the two nuclides. These difficulties have been overcome by the introduction of liquid scintillation counting, where the separation of the two isotopes is unnecessary and where the activity of each can be measured simultaneously. The instrument used in these studies was the Tri-Carb liquid scintillation spectrometer.

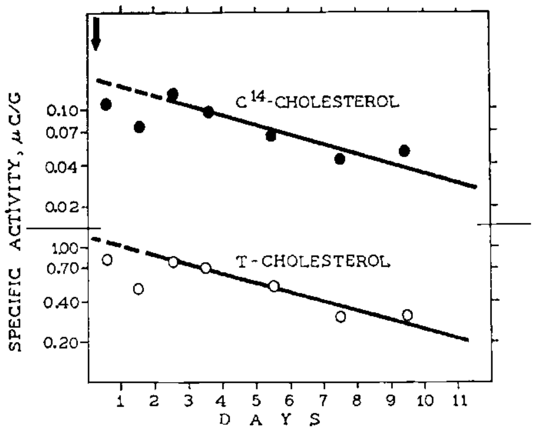

Fig. 1. Semi-log plot of cholesterol specific activity following oral administration of cholesterol-4-C^{14} and H^3-cholesterol in a normal subject.

The experiments to be reported deal with the biogenesis and metabolism of cholesterol in normal and tumor mice. Two different experimental approaches were used. In the first instance the biological decay of H^3-cholesterol and the incorporation of carboxyl-labeled acetate were studied while in other experiments the incorporation of tritium-labeled acetate and

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some other carbon-14 precursor to cholesterol was investigated. Using this double labeled technique it was not only possible to interpret changes in cholesterol concentration, but also to evaluate the contribution of various carbons sources to the sterol nucleus.

Before the experimental design was decided upon it was necessary to measure the biological stability of the tritiated cholesterol and the interval between precursor injection and the time of peak activity in the product. The answer to the first question was found in experiments with Drs. WERBIN, LEROY, *et al.* As can be seen from Fig. 1 the exponential decay of C^{14}-cholesterol labeled in the 4 position of the ring and tritium cholesterol were identical.

The second question was answered by sacrificing groups of five mice each at various time intervals. After acetate-1-C^{14} injection the results of these studies are given in Fig. 2. The maximum specific activity of liver free cholesterol occurred between 10–15 min after acetate injection.

Having determined these variables the following experimental design was decided upon. Normal mice and mice bearing either the solid Ehrlich

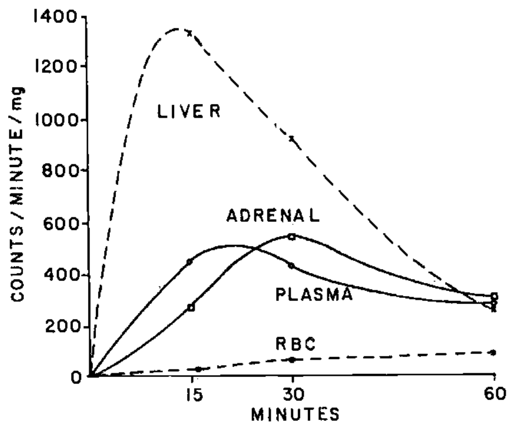

Fig. 2. The time course of lipid labeling in a normal mouse after acetate-1-C^{14} injection.

carcinoma or a lymphatic leukemia were divided into five groups of five mice each and injected with 1.0 μc of tritiated cholesterol. Three days after the injection a group of normal and pathological animals were sacrificed at 5 day intervals. Since the maximum liver specific activity of free cholesterol occurred between 10–15 min, each group of five animals previously injected with H^3-cholesterol was inoculated with acetate 15 min before decapitation and exsanguination. Their tissues were pooled, weighed, and kept at $-$ 20°C until ready for cholesterol analysis. The heparinized blood was separated immediately and the red blood corpuscles were washed twice with physiological saline before analysis. Quantitative recoveries of cholesterol were made with digitonin. It was necessary at this point to find a solvent system that would dissolve the digitonide, be suitable for the Lievermann–Burchard reaction and still possess properties suitable for liquid scintillation counting. Eastman Kodak dioxane containing 1% phosphoric acid was the system of choice. The isolated cholesterol digitonide was dissolved in the dioxane and aliquots were taken for colorimetric determination as well as radio-activity assay. The sterol containing both H^3 and C^{14} was counted simultaneously in a liquid scintillation counter by proper selection of pulse heights and voltage setting. A detailed account of the method used to isolate and assay the radioactive cholesterol is given in *Journal of Laboratory and Clinical Medicine* (July 1957), while the method used for simultaneous assay of H^3 and C^{14} compounds appeared in *Nucleonics.* (June 1957). Data from normal and tumor bearing animals were compared on the basis of disintegrations/min due to cholesterol/g tissue rather than on the basis of disintegrations/min mg cholesterol. In this way the total amount of radio cholesterol formed in an organ was measured, independent of its tissue concentration. The regression lines for H^3-cholesterol were calculated by the method of least squares. Because of the unsteady state of the tumor animal, the usual treatment of tracer data was not possible. Therefore by an alternative method which makes use of both specific activity and total activity regression curves, we were able to determine both the rate of appearance and disappearance of cholesterol in the non-steady state.

RESULTS

Rate studies from H^3*-cholesterol regression curves*—Rates calculated from the H^3-cholesterol regression line in CF-1 mice with Ehrlich carcinoma are given in the next table.

Table 1—From this table it can be seen that there is an increased appearance rate for liver, spleen, and RBC, while plasma reflects an insignificant change. The *rate of disappearance* is normal for spleen, with liver and RBC showing an increase, and the plasma having a decreased rate. The difference between these two rates is roughly the net gain of cholesterol in the organ. Except in the case of the spleen and tumor there is close agreement between quantitative levels actually measured and those calculated from rate analysis.

Figure 3—This figure gives a bar-graph indication of rates found in normal DBA mice and in mice having a lymphatic leukemia. Since there were no significant changes in quantitative levels of cholesterol, the appearance and disappearance rates were in equilibrium. The data, while suggestive of a slight lowering of rate, is not statistically significant.

Simultaneous measurement of acetate incorporation in animals from these same experiments were also conducted. The results from the CF-1 mice were so variable that no difference could be observed between normal

TABLE 1

Ehrlich Mouse Carcinoma
(Solid)

Organ	Appearance rate* mg/g tissue per day		Disappearance rate* mg/g tissue per day		Net cholesterol appearance mg/g tissue per day	
	Normal	Tumor	Normal	Tumor	Measured	Calculated
Liver	0.199	0.353	0.199	0.292	0.062	0.061
Spleen	0.267	0.413	0.267	0.262	0.043	0.151
Plasma	0.018	0.016	0.018	0.009	0.008	0.007
RBC	0.109	0.146	0.109	0.121	0.032	0.025
Tumor	—	0.177	—	0.022 (est.)	0.058	0.155

*Rates of appearance and disappearance of cholesterol in organs of mice with Ehrlich carcinoma.

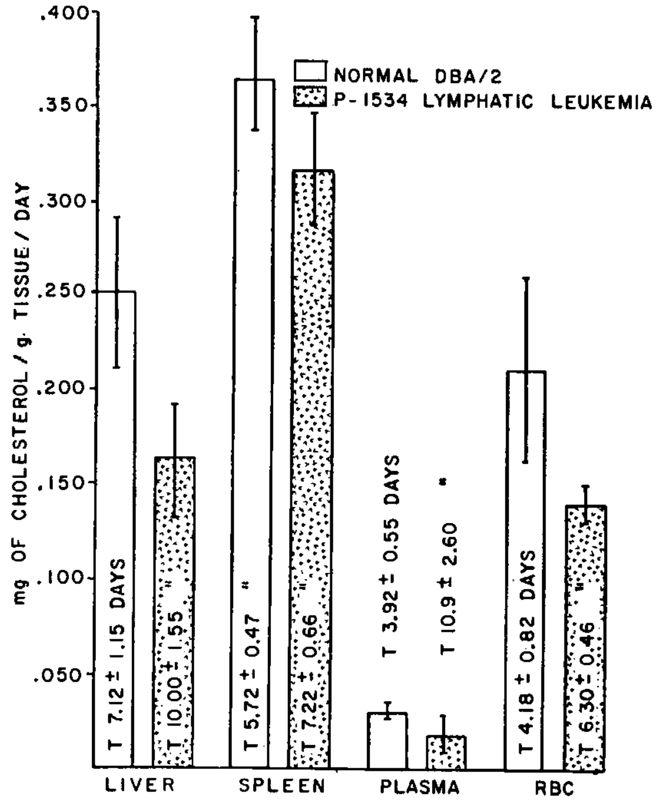

Fig. 3. Rates of cholesterol synthesis in organs of normal DBA/2 and P-1534 lymphatic leukemia mice.

and tumor. In an attempt to minimize this variation, the DBA mice were starved for 6 and 36 hr before acetate injection.

Table 2—This table shows that the contribution of liver synthesized cholesterol to peripheral tissues radioactivity could be minimized by dietary restriction. Using this technique two important facts were made evident:

TABLE 2

*Acetate-C^{14} Incorporation into Free Cholesterol**

Experimental condition	Liver	Spleen	Plasma	RBC
Control†	4480 1748 4010	1889 604 1193	29 89 50	160 24 157
P-1534 Lymphatic Leukemia				
4 days	973	1904	54	133
8 days	529	924	59	—
10 days	322	551	26	—

* Data expressed as dis/min/g tissue (wet)
† Animals were starved for 6, 36 and 6 hr respectively.
Acetate-1-C^{14} incorporation into free cholesterol.

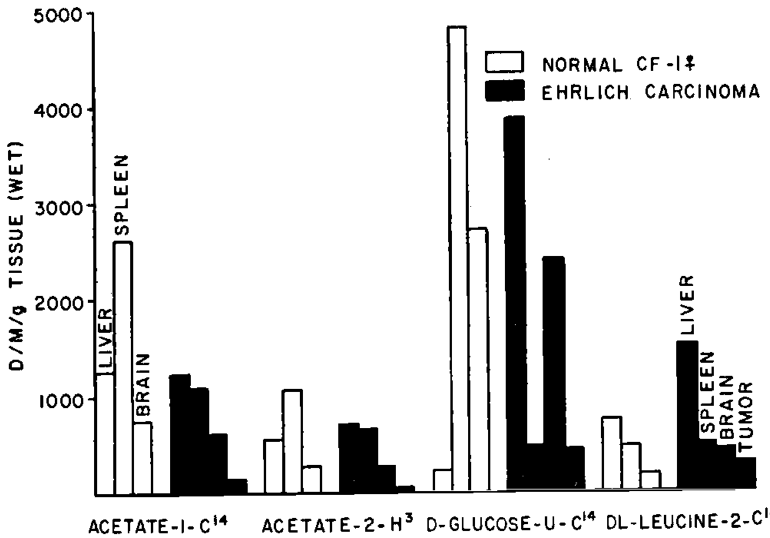

Fig. 4. Relative concentration of radioactivity of free cholesterol in tissues of normal CF-1 and Ehrlich carcinoma mice after administration of various labeled precursors.

First, it was possible to show a greater incorporation of acetate into spleenic cholesterol to that of the liver in mice infected with leukemia; and secondly, a progressive decrease of incorporation with increasing pathology of the animal was noted.

The second problem, the biogenesis of cholesterol was approached by studying the contribution of various metabolites to acetyl-CoA and thus to the cholesterol nucleus itself. It was thought that in a pathological animal an altered lipid metabolism could be reflected not only in rate changes i.e. synthesis and degradation, but also that the relative importance of various carbon sources for cholesterol synthesis could be determined.

In these experiments, tritiated acetate and carbon labeled acetate, glucose, or leucine were injected intra peritoneally and the animals sacrificed as previously mentioned. The results of these experiments are given on Fig. 4.

Figure 4—I wish to call your attention to several points: First, that the incorporation of carboxyl labeled acetate into spleen—cholesterol of Ehrlich carcinoma animals—is significantly lower than normal; that the contribution of glucose carbon to cholesterol is of interest because of the high activity found in the brain of these adult mice; and that in general, the amount of radioactivity incorporated in a particular organ is partly the function of tracer used, and does not necessarily reflect the synthetic rate of the organ.

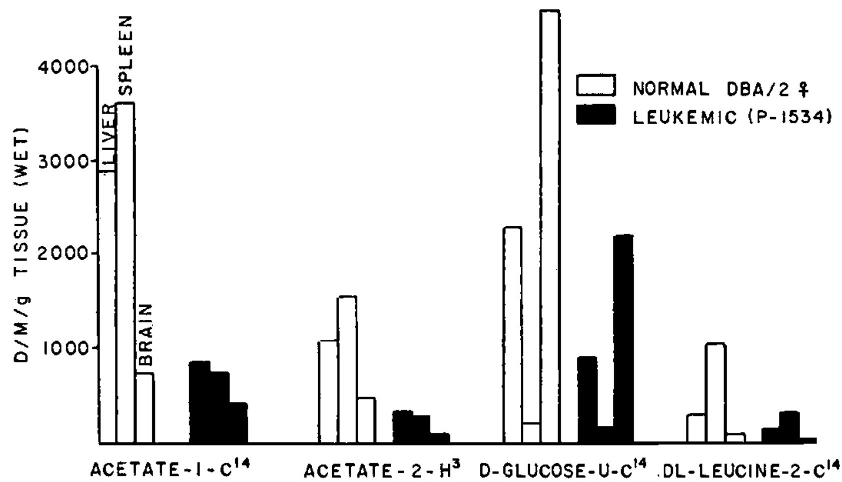

Fig. 5. Relative concentration of radioactivity of free cholesterol in tissues of normal DBA/2 and P-1534 lymphatic leukemia mice after administration of various labeled precursors.

Figure 5—The next figure with the leukemic animals shows that there is a general decrease in incorporation rates regardless of the precursor used. The high activity of the brain cholesterol following glucose injection is again significant.

In summary we have found that:

(1) Dioxane with 1% phosphoric acid was found to be a suitable solvent for both liquid scintillation counting and color assay of the dissolved cholesterol digitonide.

(2) By a discriminator-ratio method the standard error for tritium assay was less than 4% and for C^{14} less than 5%.

(3) Results on cholesterol metabolism in the unsteady state using two different experimental designs were presented. In the first instance, the biological decay of H^3 cholesterol was followed as well as the incorporation rate of acetate-1-C^{14}; In the second case, the incorporation of various metabolites labeled with tritium and/or carbon were studied.

(4) Thus, double labeled experiments using H^3 and C^{14} were made technically more feasible by the use of a liquid scintillation counter since the two nuclides could be assayed simultaneously.