THE BIOSYNTHESIS OF SQUALENE FROM ACETATE IN MAN [1]

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INTRODUCTION

The recently reported experiments of Langdon and Bloch (1, 2, 3) have furnished significant support to the hypothesis that the unsaturated hydrocarbon squalene ($C_{30}H_{50}$) is an intermediate in the pathway from acetate to cholesterol. These experiments were carried out in intact rats, as well as in rat tissue slices. Our laboratory has undertaken an investigation of the role of this hydrocarbon in metabolic pathways in the human. It has been established that the liver is the major source of plasma cholesterol (4). However, Dimter (5) and Stanger, Steiner, and Bolyard (6) were not able to isolate or identify squalene in the livers of the human and several other mammalian species. If the intermediate role of squalene is accepted, it would necessarily follow that the squalene "pool" in the liver is of very small size but turns over extremely rapidly.

The experiment reported here was designed to ascertain whether acetate is a precursor for squalene biosynthesized by the human. It has already been demonstrated that acetate is an important carbon source for cholesterol (7, 8). Since small quantities of squalene are present in human sebum, the oily secretion of the skin (9), this material was chosen as the only accessible squalene source in this experiment. The specific activities of the squalene and total cholesterol in the sebum as well as that of plasma cholesterol were measured following oral administration of acetate-2-C^{14}. The results given below demonstrate that acetate is a precursor for squalene in the human and that the squalene in human sebum is not simply derived from exogenous substances or exclusively from their degradation products.

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EXPERIMENTAL

Permission to administer acetate-2-C^{14} to a patient of limited life expectancy was obtained from the Atomic Energy Commission. The patient, a 32 year-old white female, had a functioning adrenal carcinoma. Three hundred microcuries were administered orally in a solution containing 50 mg. of sodium acetate.[2] Since experimental studies of this type in the human involve difficulties beyond those normally encountered in animal studies, data are here presented only for this one patient. As squalene is a normal component of human sebum, a demonstration in only one patient that squalene containing radiocarbon is synthesized in the human is a justifiable procedure. Sebum was collected by gently washing the skin on the arms, legs, back and scalp, using cotton swabs soaked with acetone. Precautions were taken to insure that salves or lotions, etc., were not applied to the parts used for sebum collection. There appeared to be no discomfort to the patient following this procedure.

The chloroform extraction procedure of MacKenna, Wheatley, and Wormall (10) was used after the acetone had been removed by distillation under reduced pressure in an atmosphere of nitrogen. Following the removal of the chloroform by distillation, the residue was saponified by standard procedures using aqueous-alcoholic potassium hydroxide solution. The non-saponifiable fraction was extracted with ether and, after the removal of the solvent, was then dissolved in 50 ml. of petroleum ether and poured onto a 1 cm. diameter column containing a quantity of Merck acid washed alumina 50 times the weight of the non-saponifiable portion. The hydrocarbon fractions were eluted with 250 ml. of petroleum ether (60°) and subsequent elutions with 10, 20, 30, 40, and 50 volume per cent benzene in petroleum ether were used in order to obtain the sebum cholesterol fraction.

Preparation and analysis of squalene hexahydrochloride. After removal of the petroleum ether by distillation the hydrocarbon residue was mixed with a small volume of acetone (at least ½ ml. and generally 3 times the volume of non-saponifiable fraction). Substances not soluble in this volume of acetone at room temperature were removed by centrifugation. The hexahydrochloride derivative was then prepared by treatment with dry HCl at $-5°$ for several hours. The white crystalline precipitate was washed repeatedly with absolute ether at 5°. The yields

[2] The labeled acetate was obtained from Tracerlab, Inc., after authorization from the Isotopes Division, U. S. Atomic Energy Commission.

TABLE I

Analytical data for squalene hexahydrochloride preparations*

Compound	Sample number	Remarks	Percentage by weight		
			Carbon	Hydrogen	Chlorine
Squalene hexahydrochloride	1H	Sebum-scalp	57.50	9.09	33.41
	2H	Sebum-scalp	57.22	8.78	33.59
	C	Isomer mixture	57.46	9.17	33.69
	A	113° M.P. isomer	57.42	8.93	33.58
	B	143° M.P. isomer	57.32	8.93	33.65
Calculated for $C_{30}H_{56}Cl_6$			57.23	8.97	33.79
Squalene		Redistilled	87.74	12.39	
Calculated for $C_{30}H_{50}$			87.73	12.27	

* These microanalyses were carried out by Mr. J. Alicino, Metuchen, N. J.

of $C_{30}H_{56}Cl_6$ from samples 1, 3, and 4 (Table II) were 3 to 5 mg., sufficient for radioactive assay, infra-red and x-ray powder pattern analysis. The sebum samples from the scalp yielded larger quantities of the halogen derivative. In the case of sample 1H, the weights of fat, non-saponifiable material, hydrocarbon fraction and $C_{30}H_{56}Cl_6$ were 3.1, 1.2, 0.23, and 0.027 Gm., respectively. In the case of 2H, 0.050 Gm. of $C_{30}H_{56}Cl_6$ was obtained from 2.3 Gm. of fat.

The elementary analyses for the squalene derivatives derived from the sebum or used as standards are listed in Table I. Samples A, B, and C were prepared from a redistilled sample of commercial technical grade squalene (Distillation Products Industries), obtained originally by molecular distillation of basking shark liver oil. The molecular distillation was controlled by using an iodine value as an index of purity of the squalene. The elementary analysis of the redistilled squalene agreed with the theoretical (Table I). In addition to elementary analysis, two methods were used to demonstrate that the halogen-containing derivative obtained from the hydrocarbon portion of the eluate was squalene hexahydrochloride. The infra-red spectrum of the samples derived from sebum in Table II were compared with that for standard sample C (Table I) using a double beam Perkin-Elmer spectrophotometer Model 21. Approximately 2 mg. of the $C_{30}H_{56}Cl_6$ samples from sebum (Table II), the standard sample (C) and the low melting isomeride (A-Table I) were shaken with 150 mg. of carbon disulfide. The resulting mixtures were incompletely soluble. All these mixtures (with the exception of sample 1-Table II) exhibited very similar absorption peaks, the most useful for comparison purposes at wave numbers of 1380, 1308, 1265, 1237, 1210, and 745. It was also observed that the high melting isomeride (B-Table I), mixed in the same proportion, did not furnish absorption peaks in this interval. This indicates a relatively low solubility of the 143° M.P. isomeride. The relative intensities of several peaks did not agree with the standard in the case of sample 1. The total amount of sample (about 3 mg.) did not permit any purification procedures. However, sample 1H, which covers a corresponding period of the experiment was available in sufficient quantity for precise comparison of elementary analysis, infra-red spectrum and x-ray diffraction powder photographs.

The x-ray diffraction powder photographs for the crystalline sebum squalene derivatives (Table II) were compared with standard sample C (Table I). No significant differences were detectable in all the photographs. This was also the case for the low melting derivative (Sample A-Table I). Samples A and B were prepared from C following the procedure outlined by Heilbron, Kamm, and Owens with modifications noted by Langdon and Bloch (2, 11). The powder pattern photographs were made by Dr. Benjamin Post in the X-ray Diffraction Laboratory of Brooklyn Polytechnic Institute, using a 57.32 mm. radius camera and filtered copper radiation.

Cholesterol analysis. The fractions eluted from the column with 30 to 50 per cent benzene in petroleum ether contained the sebum cholesterol. These fractions were combined and recrystallized twice from acetone yielding crystals melting at 144–46°. The cholesterol was precipitated as the digitonide for radiocarbon assay (12). The plasma cholesterol was isolated by a modification of the Sperry-Webb procedure (12).

Analysis for radiocarbon specific activity. All samples were assayed by solid plating of squalene hexahydrochloride and cholesterol digitonide in shallow stainless steel cups having a 1.63 $cm.^2$ area. Windowless gas-flow counters were used (Tracerlab Model SC-16). Correlation of observed counting rate and specific activity was made using intercalibration data obtained by the internal gas counting procedures described by Eidinoff (13, 14).

RESULTS AND DISCUSSION

The specific activities of the squalene and total cholesterol in five sebum samples are listed in Table II. The maximum squalene specific activity, 142 dpm per micromole, was found in sample 2H. The results in column 4 prove that the methyl carbon of acetate is a precursor for squalene synthesis and that sebum squalene is not simply de-

rived exclusively from exogenous sources or their degradation products.

The squalene specific activities were greater than those for the cholesterol in the sebum samples collected in the first 16 days. The plasma free cholesterol specific activities were considerably greater than those for either sebum squalene or cholesterol in the early part of the experiment and remained greater for the entire 24-day period of the experiment.

However, any attempts to compare these specific activities as functions of time in order to yield information on dynamic interrelationships meet with two serious difficulties. The first involves the collections of the sebum samples. It was not feasible to investigate the effect on the specific activities of variations in the manner, duration, and frequency of the acetone washings and of the sites used for sebum collection. The second difficulty is concerned with the physiological details of sebum formation and transport to the skin surface.

Srere, Chaikoff, Treitman, and Burstein showed that the skin and liver of the adult rat were the most active tissues with respect to the utilization of acetate for cholesterol formation *in vitro* (15). If this is assumed to be the case for the human, then the skin cholesterol specific activity may reach a maximum value within several hours after the administration of the labeled acetate, as does (Table II) plasma cholesterol (12) which is chiefly derived from cholesterol synthesized in the liver (4, 16). If it is further assumed that the squalene is an intermediate in cholesterol synthesis (2), then it is probable that both the skin squalene and cholesterol would also reach their maximum specific activities within several hours. However, the transport of a portion of these products as components of the sebum to the external surface of the skin may involve differential transfer and solubility effects. As a result, the squalene-cholesterol specific activity ratio in the sebum may not correspond to that prevailing in the interior layers of the skin at the time these materials were synthesized. Furthermore, unlabeled squalene and cholesterol present at the start of the experiment in the crypts of the glandular elements of the skin must be first removed before the newly labeled sebum is in a position to be collected. This is probably the reason for the low specific activity of the constituents in sebum collection 1 and 1H relative to the plasma free cholesterol activity in this interval. In addition to the *in situ* formation of cholesterol in the skin, cholesterol labeled with radiocarbon and synthesized in the liver is transported to the skin.

There is no clear explanation for the relatively high squalene content of sebum as compared to the as yet undetectable amounts found in human liver and spleen (5, 6, and our own unpublished experiments). Sebum contains debris of degenerated epithelial tissue. It is possible that the enzyme systems associated with cholesterogenesis are partially interrupted in those cutaneous cells which are in the process of being sloughed off into the sebum and that the squalene accumulates as a result of this metabolic alteration.

SUMMARY

1. The biosynthesis of squalene, a hydrocarbon which may be an intermediate in cholesterol synthesis, has been demonstrated in man by finding radiocarbon activity in sebum squalene after the administration of acetate-2-C^{14}.

TABLE II

Specific activity of squalene and total cholesterol in sebum samples following administration of acetate-2-C^{14}

Sample number	Source of sebum	Interval	Specific activity (*dpm/micromole*)		
			Sebum squalene	Sebum cholesterol	Plasma* free cholesterol
		(*days*)			
1	Arms, legs, back	0.3– 2.3	8 ± 1	4 ± 2	790
1H	Scalp	0– 2.4	9 ± 0.5	<1	790
2H	Scalp	2.4– 7.3	142 ± 2	98 ± 2	420
3	Arms, legs, back	9 –16	59 ± 2	34 ± 2	205
4	Arms, legs, back	19 –24	17 ± 1	72 ± 2	114

* At midpoint of sebum collection interval (column 3).

2. The specific activity of sebum squalene was compared with sebum and plasma cholesterol at several intervals over a period of 24 days and the implications of these findings are discussed.

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