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Biosynthesis of Fatty Acids in Blood and Bone Marrow of Normal and Anemic Rabbits. (32149)

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Studies involving incorporation and biosynthesis of fatty acids by blood have yielded data indicating that mixed cells of whole blood and erythrocytes(1-10), erythrocytic ghosts(10,11), platelets(12), reticulocytes(2), and leukocytes(2,5,13-16) can utilize (^{14}C)-labeled fatty acids. Most of the activity has been found in leukocytic lipids and it has also been reported that erythrocytes can synthesize a variety of long-chain saturated and polyenoic acids(1,7,10,13,15,16). Plasma lipids have been found to be labeled in experiments employing plasma as suspending medium for blood cells(1,3,5,6).

This report describes studies which were conducted on the biosynthesis of fatty acids by cellular elements of blood and bone marrow tissue from normal and phenylhydrazine-treated rabbits. In addition, data are given on the activity found in the lipids of plasma which served as suspending medium.

Materials and methods. Precursor fatty acid. Sodium (2-^{14}C) acetate was obtained from Radiochemical Centre, Amersham, England. It was made up to a concentration of 10 μc/0.1 ml with 0.85% NaCl solution and dispensed at the rate of 1-2 μc/ml of blood and bone marrow cell suspension.

Preparation of blood. Blood was collected in heparin by exsanguination of 2- to 3-month-old New Zealand White rabbits. Packed cell volumes averaged 42% and reticulocytes remained below 0.5% in blood from normal rabbits.

In order to produce an artificial anemia, rabbits were injected subcutaneously with an aqueous solution of phenylhydrazine hydrochloride at the rate of 4 mg/kg body weight/day for 7-9 days and bled 2 days after the last injection. Percentage of reticulocytes ranged from 69-93% and packed cell volumes from 22-34%.

Fifty ml of blood were used for incubation with sodium (2-^{14}C) acetate.

Bone marrow preparations. Bone marrow was collected from rabbits that were used to provide material for the blood studies. Tissues obtained from 2-8 femurs were shaken in plasma at 38°C for 15 min to separate and suspend the cells. The suspension was recovered by centrifugal sedimentation, discarding the floating fatty tissue and the sedi-

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TABLE I. Representative Labeling of the Fatty Acids of Lipids from Cells and Plasma of Normal Rabbit Blood Incubated for 5 and 22.5 Hours. Conditions: 50 ml of whole blood, 1 μC sodium (2-^{14}C) acetate/ml, temperature 38°.

Incubation time	Lipid	Specific activity (mμC/25 mμg fatty acid) 10:0	12:0	14:0	16:0	18:0	18:1	18:2
5 hr*	Cellular free fatty acid		51.2	44.8	12.8	trace		
	Plasma free fatty acid	108.8	134.4	227.2	64.0	12.8	3.2	
	Cellular triglyceride		trace	108.8	22.4	12.8		
	Cellular phospholipid					trace		
22.5 hr†	Plasma free fatty acid	236.8	185.6	332.8	32.0	25.6	6.4	trace
	Cellular triglyceride			trace	trace			
	Cellular phospholipid					trace		

No. of determinations: * 7; † 3.

mented cells suspended in 50 ml of rabbit plasma. Normal plasma was used for suspending tissues from normal rabbits, whereas plasma from phenylhydrazine-treated rabbits was employed as suspending medium for cells of treated animals.

Bone marrow smears from both groups of animals showed mainly normoblastic cells. However, tissues from treated rabbits appeared to be extremely fluid and active throughout the shaft of the femur. Normal tissues were not fluid and only showed activity near the epiphyses.

Incubation. Versene, penicillin and streptomycin were added at the rate of 0.05 ml of a 0.01% solution, 5,000 I.U. and 7,450 I.U., respectively, per 5 ml of incubation mixture. Aseptic technique and sterile glassware were employed throughout the study. Flasks were plugged with sterile cotton wool and incubated between 5 and 22.5 hr in an agitating water-bath at 38°C.

Lipid extraction and characterization. After incubation the samples were centrifuged and the cells and clarified plasma recovered. In centrifuging bone marrow cells there was usually a layer of floating cells (low density) which were also recovered as separate samples from the sedimented cells. All cells were centrifugally-washed twice with 0.85% NaCl solution. Total lipids from the suspending medium and cells were each extracted overnight with chloroform:methanol (2:1, v/v) and recovered as described by Folch *et al*(17).

The total lipid extracts in chloroform were dried over Na_2SO_4, reduced in volume and the classes separated by thin-layer chromatography on Silica Gel G in a solvent system consisting of petroleum ether, diethyl ether and formic acid (84:15:1, v/v/v)(18).

Plates were sprayed with 0.2% 2′,7′-dichlorofluorescein and the lipid classes detected by fluorescence under ultraviolet light. Zones containing each class were scraped from the plates, placed in columns and eluted from the adsorbent using diethyl ether for triglycerides, free fatty acids and sterol esters and methanol:chloroform (2:1, v/v) for phospholipids. The purified lipids were taken to dryness and transmethylated using a modification of the method described by Stoffel *et al*(19) in which H_2SO_4 was substituted for HCl.

Resulting methyl esters were separated in an ethylene glycol adipate column, 4 feet long, which was maintained at 180°C. Both mass and radioactivity were monitored with a radiochemical gas chromatograph constructed as described by James and Piper (20). Specific activities were expressed as mμC/25 mμg fatty acid after relating mass and radioactive peak areas.

Results. Normal blood. The results of short and long term incubations on the biosynthesis of fatty acids by normal rabbit blood cells suspended in plasma as whole blood are given in Table I. After 5 hours both the free fatty acid and triglyceride fractions of the cells and the free fatty acids of plasma showed labeling. There was only a trace of activity in the cellular phospholipid. The data indicated production of free fatty acids by the cellular elements and passage out into plasma.

Incubation of normal rabbit blood for 22.5

TABLE II. Representative Fatty Acid Synthesis in Blood of Rabbits Treated with Phenylhydrazine. Conditions: 50 ml of whole blood, 2 μC sodium (2-^{14}C) acetate/ml, temperature 38°, incubation time 5 hours.

Fraction	Lipid	Specific activity (mμC/25 mμg fatty acid) 10:0	12:0	14:0	16:0	18:0
Cells*	Free fatty acid		32.0	64.0	6.4	
	Triglyceride		9.6	76.8	9.6	3.2
	Phospholipid					trace
Plasma†	Free fatty acid	80.0	358.4	640.0	12.8	48.0

No. of determinations: * 3; † 3.

hours produced higher levels of labeling in the free fatty acid fraction of plasma, whereas activity in cellular triglyceride and phospholipid decreased to a low level (Table I). Labeled free fatty acids were not found in the cells. This decrease in labeling of cellular lipids suggested turnover of the triglyceride and phospholipid pools and the continuous secretion of free fatty acids by the cells out into the plasma.

Anemic blood. When compared to normal rabbits (Table I) blood cells from anemic animals did not appear to have their fatty acid synthetic abilities significantly changed (Table II) even though the samples contained large numbers of reticulocytes.

Normal bone marrow. Normal bone marrow showed much more rapid synthesis of fatty acids than the whole blood incubations previously described (Tables I, II). In 5 hours, labeling of a variety of saturated and unsaturated fatty acids occurred in the free fatty acid, triglyceride, and phospholipid fractions of the sedimented cells and in the triglyceride fraction of the floating cells (Table III). There were also high levels of labeling in plasma free fatty acids and to a lesser extent in triglycerides and phospholipids. The data demonstrated that there was considerable export of not only free fatty acids but also of triglycerides and phospholipids by bone marrow cells out into the plasma. However, in 22 hours activities had generally decreased (Table IV) indicating reexchange of lipid between plasma and cells.

Bone marrow in anemia. When compared to bone marrow systems of normal rabbits (Tables III, IV) synthesis of fatty acids was found to be decreased in phenylhydrazine anemia (Table V). Furthermore, plasma triglycerides and phospholipids and cellular triglycerides were not found to be labeled.

Sterol esters recovered from all blood, bone marrow, and plasma lipids throughout this investigation did not exhibit incorporation of acetate.

A number of experiments were also conducted employing plasma alone and normal erythrocytes which were purified by washing several times by centrifugation in plasma and incubated with sodium (2-^{14}C) acetate. Synthesis of fatty acids in the lipid classes of plasma or purified erythrocytes was not detected.

The percentage of radioactivity taken up by the total lipid in 5 hours averaged 2.2% for normal blood cells, whereas for normal bone marrow cells the value was 3.1%. Values for anemic blood and bone marrow cells were generally slightly lower.

Discussion. Leukocytes and platelets(2,12) have been incriminated as the main cellular elements which contribute to fatty acid biosynthesis in blood. The mature erythrocyte has been relegated to a minor biosynthetic role, if any(12,13), although it can incorporate a variety of fatty acids(11). Young erythrocytes(12,13) and reticulocytes(2,12) have been shown to be capable of synthesizing and incorporating fatty acids but their contribution is minor when compared to leukocytes(2,12).

The degree of activity and the number of fatty acids synthesized by blood cells from sodium (2-^{14}C) acetate were lower than those given by other workers who studied the capabilities of cells from human(1,3,5,7,8,12) and fowl blood(9,13). Altman(11) confirmed that the incorporation of (2-^{14}C) acetate takes place in stroma of rabbit erythrocytes. However, activities of lipids in

erythrocytes were not determined in that study.

Altman(11) reported that plasma was not necessary for incorporation of acetate to take place in erythrocytic stroma although large amounts of activity were found in the lipids of plasma when it was employed as suspending medium. There is ample documentation of the influence of plasma proteins in binding fatty acids which are produced by a variety of cells(22-24). When albumin is present there is a greater production of fatty acids. Protein serves to facilitate transfer of fatty acids from cells to medium, thereby preventing accumulation of these substances in actively-metabolizing cells(21,25). Fur-

TABLE III. Representative Distribution of Labeled Fatty Acids in the Lipids of the Medium and Cells of Normal Bone Marrow in Plasma. Conditions: Volume of medium 50 ml, 2 μC sodium ($2\text{-}^{14}C$) acetate/ml, temperature 38°, incubation time 5 hr.

		Specific activity (mμC/25 mμg fatty acid)											
Cells and medium	Lipid	10:0	12:0	14:0	16:0	18:0	20:0	14:1	16:1	18:1	18:2	Unknown	Conj. 18:2
Sedimented cells*	Free fatty acid	64.0	86.4	390.4	70.4	124.8		192.0	160.0	38.4			
	Triglyceride	198.4	150.4	153.6	160.0	80.0	636.8			80.0	44.8	240.0	192.0
	Phospholipid			953.6	512.0	384.0	640.0			150.4	12.8	2080.0	579.2
Floating cells†	Triglyceride	51.2	112.0	448.0	169.6	38.4			112.0	54.4			
Plasma‡	Free fatty acid	368.0	1056.0	1280.0	1888.0	400.0	1120.0		521.6	192.0			2432.0
	Triglyceride		54.4	592.0	118.4	86.4			144.0	12.8			
	Phospholipid		trace	150.4	3.2	1.28							

No. of determinations: * 4; † 2; ‡ 4.

TABLE IV. Representative Distribution of Labeled Fatty Acids in the Lipids of the Medium and Cells of Normal Bone Marrow in Plasma. Conditions: Volume of medium 50 ml, 2 μC sodium ($2\text{-}^{14}C$) acetate/ml, temperature 38°, incubation time 22 hr.

		Specific activity (mμC/25 mμg fatty acid)											
Cells and medium	Lipid	10:0	12:0	14:0	16:0	18:0	20:0	14:1	16:1	18:1	18:2	Unknown	Conj. 18:2
Sedimented cells*	Free fatty acid			137.6	28.8								
	Triglyceride		32.0	99.2	22.4	163.2	22.4	76.8	118.4	9.6		512.0	
	Phospholipid			406.4	9.6	38.4	736.0		86.4	22.4	80.0	544.0	960.0
Floating cells†	Triglyceride			25.6	6.4	3.2				1.28			
	Phospholipid			134.4	28.8	35.2							
Plasma‡	Free fatty acid	137.6	1120.0	608.0	80.0	60.8	4448.0		588.8	22.4	19.2	736.0	310.4
	Triglyceride			trace	trace	trace							
	Phospholipid			"	"	"							

No. of determinations: * 3; † 2; ‡ 3.

TABLE V. Representative Distribution of Labeled Fatty Acids in the Lipids of the Medium and Cells of Bone Marrow Collected from Rabbits Made Anemic with Phenylhydrazine and Incubated in Plasma. Conditions: Volume of medium 50 ml, 2 μC sodium (2-^{14}C) acetate/ml, temperature 38°, incubation time 5 hours.

		Specific activity (mμC/25 mμg fatty acid)						
Cells and medium	Lipid	10:0	12:0	14:0	16:0	18:0	20:0	Unknown
Sedimented cells*	Free fatty acid		trace	16.0	1.28	trace		
	Phospholipid				trace	16.0	102.4	
Plasma†	Free fatty acid	704.0	288.0	384.0	38.4	736.0	12.8	800.0

No. of determinations: * 3; † 3.

thermore, total free fatty acid concentration depends on the net difference between production of fatty acids by lipolysis and transfer across the cell membrane and esterification. Protein-binding of fatty acids in the medium then permits lipolysis to proceed further and accumulation in cells does not occur(21).

Miras *et al*(14) have reported incorporation of (1-^{14}C) acetate into the phospholipids of human leukocytes to be at a lower rate than for neutral lipids. This may explain the relatively higher levels of active fatty acids in the free fatty acid and triglyceride fractions found in rabbit blood cells. Furthermore, it has been confirmed that leukocytes are responsible for most of the active fatty acids of the phospholipids found in human blood(2).

The major differences between the biosynthetic capabilities of bone marrow and blood cells were that bone marrow cells produced labeled free fatty acids, triglyceride, and phospholipid and at higher levels as demonstrated by the activity found in plasma (Table III). Only labeled free fatty acid was detected in the plasma of blood cell systems (Table I).

Phenylhydrazine is known to be a hemolytic agent and is administered to humans for polycythemic disorders(26). It destroys only mature erythrocytes and newly formed reticulocytes are spared the action of the drug. Enhanced stimulation of bone marrow activity and reticulocytosis occurs as a result of the destructive anemia(27).

O'Donnell *et al*(28) produced anemia in rabbits with phenylhydrazine and studied the biosynthesis of lipids from (1-^{14}C) acetate in reticulocytes. The specific activities found in these lipids were greater than those observed in normal blood cells but lipid class fractionation and assay of individual fatty acids were not conducted. In the present studies employing whole blood from anemic rabbits having high numbers of reticulocytes, the data indicated little difference in the synthesis of fatty acids (Tables I, II). However, with bone marrow cells from phenylhydrazine-treated rabbits the biosynthesis of fatty acids was decreased (Table V). The mechanism involved is obscure but this decrease may result from a change in the types of cells which produce fatty acids(15) or it may be due to alteration in the rate of metabolism brought about by the drug.

It was anticipated that a greater degree of fatty acid biosynthesis would occur in samples from anemic rabbits because of the large numbers of reticulocytes(28) and young erythrocytes in blood and nucleated cells from bone marrow. Reticulocytes(2,12) and young erythrocytes(12) have been shown to be capable of synthesizing fatty acids to a greater degree than mature erythrocytes. However, this was not the case in the present studies. Phenylhydrazine may be inhibitory to fatty acid biosynthesis. This hypothesis can be tested by inducing an anemia by withdrawal of large quantities of blood in order to stimulate the hemopoietic system to produce reticulocytes and young erythrocytes in abnormal numbers.

The data on fatty acid biosynthesis in phenylhydrazine anemia present questions for further study. Phenylhydrazine may inhibit synthesis of triglycerides in bone marrow cells. The effect of phenylhydrazine on lipolysis and on the elaboration of phospholipids and triglycerides into the suspending medium needs to be investigated.

It is interesting to speculate further on

the effect of phenylhydrazine on fatty acid biosynthesis, as to whether this change occurs *in vivo* as a direct effect on the overall metabolic activities or whether it is an *in vitro* effect because of residual phenylhydrazine in the cells and plasma obtained from the anemic animal. It would be prudent to test these questions *in vitro* by incubating normal blood and bone marrow with added phenylhydrazine.

It is evident from the results that free fatty acids are elaborated into plasma from cells at a rapid rate *in vitro,* whereas their return to the cell is relatively slower. Triglycerides and phospholipids appear to have a much lower turnover than free fatty acids.

Summary. Blood and bone marrow cells from normal rabbits and rabbits made anemic with phenylhydrazine were incubated with sodium (2-^{14}C) acetate. The newly-synthesized fatty acids in lipids of both cells and suspending medium were detected by radiochemical gas chromatography. In normal blood, labeled fatty acids were found in the free fatty acid, triclyceride, and phospholipid fractions, whereas plasma contained activity only in the free fatty acid fraction. There were no major differences in lipid synthesis by blood from anemic rabbits. Normal bone marrow cells rapidly synthesized fatty acids which were found in free fatty acid, triglyceride, and phospholipid fractions. Similar activities were detected in these 3 lipid fractions of plasma. Incubation of normal bone marrow for 22 hours indicated that labeling of plasma triglycerides and phospholipids had decreased demonstrating reexchange with cellular lipids. There was decreased lipid synthesis in bone marrow cells of anemic rabbits. Labeled fatty acids were found only in free fatty acid and phospholipid fractions, whereas activity was detected only in free fatty acids of plasma.

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