activities of 57 and 63% respectively as compared with that of the native protein.

SUMMARY

1. The preparation and spectra of native, reconstituted proto-, meso- and deuterio-myoglobin are described.

2. The rates of combination of these proteins with carbon monoxide have been measured. In most cases the rate for the reconstituted proto-myoglobin was lower than that for the native protein.

3. The relative rates for reconstituted proto-, meso- and deuterio-myoglobin were 1:1:3.5.

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REFERENCES

- Bowen, W. J. (1948). *J. biol. Chem.* **176**, 747.
 Bowen, W. J. (1949). *J. biol. Chem.* **179**, 235.
 Britton, H. T. S. & Welford, G. (1937). *J. chem. Soc.* p. 1848.
 Clark, W. M. (1928). *The Determination of Hydrogen Ions*, 3rd ed., p. 200. Baltimore: Williams and Williams.
 Drabkin, D. L. (1942). *J. biol. Chem.* **146**, 605.
 Drabkin, D. L. (1945). *J. biol. Chem.* **158**, 721.
 Drabkin, D. L. (1947). *J. biol. Chem.* **171**, 409.
 George, P. & Irvine, D. H. (1952). *Biochem. J.* **52**, 511.
 Gibson, Q. H. (1956). *J. Physiol.* **134**, 112.
 Gibson, Q. H. & Smith, M. H. (1957). *J. Physiol.* **136**, 27P.
 Gjessing, E. G. & Sumner, J. B. (1942). *Arch. Biochem.* **1**, 1.
 Hill, R. & Holden, H. F. (1926). *Biochem. J.* **20**, 1326.
 Hogness, T. R., Zscheile, F. F., jun., Sidwell, A. E., jun. & Barron, E. S. G. (1937). *J. biol. Chem.* **118**, 1.
 Kendrew, J. C., Bodo, G., Dintzis, H. M., Parrish, R. G. & Wyckoff, H. (1958). *Nature, Lond.*, **181**, 662.
 Kiese, M. & Kaeske, H. (1942). *Biochem. Z.* **312**, 121.
 Lemberg, R. & Legge, J. W. (1949). *Hematin Compounds and Bile Pigments*. New York: Interscience Publishers Inc.
 Millikan, G. A. (1936). *Proc. Roy. Soc. B*, **128**, 366.
 Rossi-Fanelli, A. & Antonini, E. (1957). *Arch. Biochem. Biophys.* **72**, 243.
 St George, R. C. C. & Pauling, L. (1951). *Science*, **114**, 629.
 Smith, M. H. (1959). *Biochem. J.* **73**, 90.
 Sørensen, S. P. L. (1909). *Biochem. Z.* **21**, 131.
 Theorell, H. (1932). *Biochem. Z.* **252**, 1.
 Theorell, H. (1934). *Biochem. Z.* **268**, 55.
 Theorell, H. & Åkeson, A. (1955). *Ann. Acad. Sci. fenn.* **IIa**, **60**, 303.
 Theorell, H., Bergstrom, S. & Åkeson, A. (1942). *Ark. Kemi Min. Geol.* **A16**, no. 13.
 Theorell, H. & de Duve, C. (1947). *Arch. Biochem.* **12**, 113.
 Warburg, O. & Negelein, E. (1932). *Biochem. Z.* **244**, 9.

The Lipids of Whole Blood

1. LIPID BIOSYNTHESIS IN HUMAN BLOOD *IN VITRO*

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In recent years the erythrocyte has been shown [Caffrey, Tremblay, Gabrio & Huennekens (1956), and Lowy, Ramot & London (1958)] to possess at least some of the normal metabolic pathways, contrary to the older view which considered the cell simply as an inert carrier of haemoglobin (for a recent review see Prankherd, 1955). Little detailed study, however, has been made of lipid turnover, though Lovelock (1955) suggested on the basis of physical measurements that the structural integrity of the erythrocyte is maintained by its own metabolic activity. Altman, Watman & Salomon (1951) described the incorporation *in vivo* of [*Me*-¹⁴C]acetate into the stroma of the rabbit erythrocyte and suggested that metabolically active lipid constituents of the stroma (with a life span of 3-4 days) exchange with chemically identical plasma components. This type of ex-

change had previously been demonstrated by Muir, Perrone & Popják (1951) to occur with erythrocyte cholesterol. Altman (1953) reported the incorporation *in vitro* of [*carboxy*-¹⁴C]acetate into the stroma of the rabbit erythrocyte during the incubation, either of whole blood or of erythrocytes in saline; approximately 1.9% of the total activity was found in the stroma after 6 hr. Activity of a comparable order was also found in the fatty acids of the plasma lipids. Similar incorporations *in vitro* of labelled acetate were demonstrated to occur in human blood by Altman & Swisher (1954).

We have extended this work and have demonstrated the ability of the cellular components of blood to synthesize a wide variety of fatty acids, to incorporate these acids into both triglycerides and phospholipids and to exchange both lipids with

certain of the plasma lipoproteins. Preliminary accounts of this work have already been published (James, Lovelock & Webb, 1957, 1958).

EXPERIMENTAL

Blood was withdrawn from volunteers by venepuncture directly into sterile citric acid-sodium citrate-dextrose (Lovelock, 1955). All incubations were carried out in sterile apparatus in the presence of antibiotics. In a typical experiment 10 ml. of whole blood was added to 1.5 ml. of citric acid-sodium citrate-dextrose with the following additions: [$Me-^{14}C$]acetate ($10\mu\text{C}$; 0.73 mg.), penicillin (1000 units), streptomycin (1000 units) and ethylenediaminetetra-acetic acid ($50\mu\text{g}$.) and incubated in a centrifuge pot on a roller-tube apparatus at 37° in air.

At the end of the incubation period (normally 5 hr.) the red and white cells were removed by centrifuging and then washed with 3 ml. of 0.9% NaCl soln., the washings being added to the plasma. The cells were resuspended in 0.9% NaCl and added in a thin stream to 20 vol. of stirred ether-ethanol (1:2, v/v). The extraction was allowed to proceed at room temperature for 18 hr. The plasma was divided into two fractions by the addition of dextran sulphate, by the method of Oncley, Walton & Cornwell (1957). The precipitate produced consisted of β -lipoprotein and probably some of the chylomicra or the lower-density lipoproteins. The soluble portion is referred to in this paper as the α -lipoprotein fraction. The precipitate was removed by centrifuging and both plasma fractions were extracted with ether-ethanol overnight.

After filtration, the ether-ethanol lipid solution was evaporated to dryness *in vacuo* and extracted twice with acetone. The insoluble material is referred to as phospholipid and the soluble portion as neutral fat. Phosphorus analyses showed that the soluble fraction contained very little phospholipid.

In one experiment the neutral fat from both α -lipoproteins and erythrocytes was separated into cholesterol esters, triglyceride and free sterol by chromatography in a liquid-solid system, with silica gel (Mallinckrodt) as the adsorbent and with the solvent system described by Hirsch & Ahrens (1958). The neutral fat (89 mg.) was applied to the column (1.1 cm. diam. by 30 cm.; 18 g. of adsorbent) in a small volume of light petroleum (b.p. $40-60^\circ$). The cholesterol esters (39 mg.) were eluted with 350 ml. of 1% (v/v) ether-light petroleum and 60 ml. of 4% (v/v) ether-light petroleum, the triglycerides with 350 ml. of 4% (v/v) ether-light petroleum and the free sterols with 10% (v/v) ether-light petroleum. Two further fractions, possibly consisting of diglyceride and small amounts of phospholipid, were eluted with ether and then with acetone. Both fractions contained bound fatty acids, as did the free sterol fraction. The lipid fractions were saponified by heating under reflux with methanolic 10% KOH for 2 hr. At the end of this period the methanol was removed *in vacuo*, water was added and the non-saponifiable material was removed by extraction (twice) with light petroleum. After acidification with 5N- H_2SO_4 the long-chain fatty acids were extracted with ether. The solution of fatty acids in ether was dried with anhydrous Na_2SO_4 , evaporated to dryness and esterified by refluxing with dry methanolic 0.8N-HCl for 2 hr. The procedures of application of the sample to the gas chromatogram and counting adopted for the isolated

fractions have been described previously (James, Peeters & Laurysens, 1956). Where complete separation of acids was not achieved, e.g. linoleic acid and oleic acid, only parts of the zones were collected so that contamination of fractions was avoided. The gas chromatogram used for most of the work had Apiezon M grease as the stationary phase so that linoleic acid and linolenic acid were not separated. The counting was carried out with a conventional end-window counter registering 50 000 counts/min. with a standard of $1\mu\text{C}$ of ^{14}C . Acids of as yet unknown structure are included in this study (see James & Webb, 1957).

The [$Me-^{14}C$]acetate used in these experiments was checked for the presence of higher fatty acids of microbiological origin by adding $1\mu\text{C}$ to a sample of unlabelled plasma lipids. The mixture was subjected to the normal process of extraction, saponification and esterification and the methyl esters of the fatty acids isolated were separated on the gas chromatogram. No activity could be detected in any of the acids.

Incubation in 0.9% sodium chloride. Cells were removed from whole blood by centrifuging, washed twice with 0.9% NaCl and resuspended in this solution with the same additions as described for whole blood. At the end of 5 hr. the red and white cells were removed, washed and extracted with ether-ethanol as before. The 0.9% NaCl supernatant was also extracted. No labelled long-chain fatty acids could be found in this supernatant.

RESULTS

In the early experiments, in which [$Me-^{14}C$]acetate was incubated with sterile whole blood for 5 hr., isolation of the erythrocyte and plasma phospholipids and neutral fat showed appreciable radioactivity in all fractions, as described by Altman (1953). Isolation of the fatty acids from these lipids showed most of this activity to be due to the long-chain fatty acids. In Tables 1 and 2 are shown comparisons of specific activities of the major fatty acid components of these lipids, in experiments with blood from three subjects. Tables 3 and 4 give the fatty acid compositions of the lipids studied. Maximal activity was found in the plasma neutral fat but this could not be due to any synthetic activity of the plasma itself since incubation of labelled acetate with blood after removal of the red and white cells produced very little incorporation into either neutral fat or phospholipid. (This residual incorporation could be due to the platelets remaining.) This synthesis of long-chain acids is therefore carried out by the red and possibly the white cells, which produce both neutral fat and phospholipid, which are then secreted into the plasma.

Reference was made in an earlier publication (James & Lovelock, 1956) to the great variety of fatty acids in the human erythrocyte. All the odd-number straight- and branched-chain acids and unsaturated acids up to a chain length of 20 carbon atoms that exist as minor components were labelled as well as those described in Table 1, but

in this paper attention is directed only towards the major components.

It cannot yet be stated with certainty whether the activity of the mixed peak of linoleic acid and linolenic acid is due to labelling of one or both acids. However, the fact that the activity of arachidonic acid in general follows that of the two-component peak suggests that linoleic acid is labelled.

In two of the experiments reported above, sufficient material was available for separation of the plasma lipoproteins into two main groups by precipitation of the β -lipoprotein with dextran sulphate. The two fractions were extracted and the extract was split into neutral fat and phospholipid. In Table 5 is given the fatty acid composition of the four fractions obtained from two blood samples.

Table 1. *Comparison of specific activities of isolated fatty acids from erythrocyte lipids*

—, Not determined; 0, zero value; m.u., mono-unsaturated.

Acid	(Counts/min./mg.)					
	Erythrocyte neutral fat		Erythrocyte phospholipids			
	Subject 1	Subject 3	Subject 1	Subject 2	Subject 3	
<i>n</i> -C ₁₀	—	325	—	—	255	
<i>n</i> -C ₁₂	—	—	—	—	—	
<i>n</i> -C ₁₄	64	660	67	174	506	
m.u. C ₁₆	—	25	—	—	69	
<i>n</i> -C ₁₈	176	132	31	52	41	
Di- + tri-unsat. C ₁₈	0	0	0	5	34	
m.u. C ₁₈	104	60	81	27	37	
<i>n</i> -C ₁₈	400	203	87	54	87	
Unsat. 'C ₁₉ A'	—	—	—	13	78	
Arachidonic	—	—	—	7	18	
Poly-unsat. C ₂₀	—	—	43	70	84	

Table 2. *Comparison of specific activities of isolated fatty acids from plasma lipids*

—, Not determined, as quantity was too small; 0, zero value; m.u., mono-unsaturated.

Acid	(Counts/min./mg.)					
	Plasma neutral fat			Plasma phospholipid		
	Subject 1	Subject 2	Subject 3	Subject 1	Subject 2	Subject 3
<i>n</i> -C ₁₀	—	—	1300	—	—	1070
<i>n</i> -C ₁₂	1620	1365	1030	—	310	760
<i>n</i> -C ₁₄	661	416	520	89	185	612
m.u. C ₁₆	275	63	102	—	9	129
<i>n</i> -C ₁₆	682	590	180	25	54	570
Di- + tri-unsat. C ₁₈	36	42	61	7	27	31
m.u. C ₁₈	170	68	377	32	22	40
<i>n</i> -C ₁₈	284	272	312	43	98	122
Unsat. 'C ₁₉ A'	—	—	—	—	—	—
Arachidonic	—	—	—	12	20	21
Poly-unsat. C ₂₀	—	—	—	31	41	83

Table 3. *Fatty acid composition of erythrocyte lipids in Table 1*

—, Not determined, as quantity was too small; 0, zero value; trace, less than 1%; m.u., mono-unsaturated. Composition is given as percentages. Major components only are listed.

Acid	Erythrocyte neutral fat		Erythrocyte phospholipid		
	Subject 1	Subject 3	Subject 1	Subject 2	Subject 3
<i>n</i> -C ₁₀	0	0.6	0	0	0
<i>n</i> -C ₁₂	1.1	2.2	0	0.1	0.2
<i>n</i> -C ₁₄	17.3	5.9	2.5	1.9	1.2
m.u. C ₁₆	3.2	6.0	Trace	1.10	1.6
<i>n</i> -C ₁₆	15.2	22.6	25.2	23.7	23.0
Di- + tri-unsat. C ₁₈	21.1	11.4	9.1	12.3	8.6
m.u. C ₁₈	29.1	28.8	17.0	19.0	25.5
<i>n</i> -C ₁₈	5.7	10.7	16.6	19.2	17.7
Unsat. 'C ₁₉ A'	Trace	Trace	Trace	5.7	2.2
Arachidonic	8.3	7.4	22.3	12.3	10.0
Poly-unsat. C ₂₀	Trace	Trace	4.1	2.4	4.1

Table 4. *Fatty acid composition of plasma lipids in Table 2*

—, Not determined, as quantity was too small; 0, zero value; trace, less than 1%; m.u., mono-unsaturated. Composition is given as percentages. Major components only are listed.

Acid	Plasma neutral fat			Plasma phospholipid		
	Subject 1	Subject 2	Subject 3	Subject 1	Subject 2	Subject 3
<i>n</i> -C ₁₀	0.2	0	0.2	0	0	0.1
<i>n</i> -C ₁₂	0.9	0.7	0.6	Trace	0.3	0.4
<i>n</i> -C ₁₄	10.2	10.9	4.3	2.9	2.6	2.0
m.u. C ₁₆	2.2	5.4	4.5	1.7	2.4	4.0
<i>n</i> -C ₁₆	21.2	22.4	26.0	28.0	23.3	17.0
Di- + tri-unsat. C ₁₈	12.5	11.5	6.6	17.2	19.5	18.7
m.u. C ₁₈	25.1	30.1	43.8	16.0	19.5	32.3
<i>n</i> -C ₁₈	8.3	7.4	10.7	14.4	14.1	8.4
Unsat. 'C ₁₉ A'	Trace	Trace	—	Trace	0.6	Trace
Arachidonic	8.0	2.4	—	11.6	7.7	9.4
Poly-unsat. C ₂₀	4.0	Trace	—	3.4	5.6	5.0

Table 5. *Fatty acid composition of lipids of plasma α- and β-lipoproteins*

—, Not determined, as quantity was too small; 0, zero value; trace, less than 1%; m.u., mono-unsaturated; br., branched (*iso*- or *ante-iso*). Composition is given as percentages. Major components only are listed.

Acid	α-Lipoprotein				β-Lipoprotein		
	Neutral fat		Phospholipid		Neutral fat + phospholipid Subject 1	Neutral fat Subject 2	Phospholipid Subject 2
	Subject 1	Subject 2	Subject 1	Subject 2			
m.u. C ₁₂	0	0.3	0.3	0	0.6	0	0.5
<i>n</i> -C ₁₂	0.4	0.8	0.2	0	0.5	2.2	0.5
m.u. C ₁₄	1.4	2.5	0.8	5.1	2.2	6.8	2.8
<i>n</i> -C ₁₄	2.5	3.7	0.8	3.6	2.1	3.6	0.9
<i>n</i> -C ₁₅	1.1	1.1	1.0	Trace	0.9	Trace	Trace
m.u. C ₁₆	4.9	5.2	0.8	7.1	3.2	5.0	2.6
<i>n</i> -C ₁₆	18.5	25.7	29.6	37.8	24.0	19.8	31.3
br. C ₁₇	2.2	1.8	1.3	Trace	Trace	1.1	4.3
<i>n</i> -C ₁₇	Trace	Trace	1.4	Trace	Trace	Trace	Trace
Di- + tri-unsat. C ₁₈	16.3	13.4	18.1	14.8	7.9	10.5	10.2
m.u. C ₁₈	38.8	35.8	15.1	15.3	16.8	18.4	17.4
<i>n</i> -C ₁₈	8.1	7.6	16.4	16.4	23.1	10.7	18.5
Unsat. 'C ₁₉ A'	1.8	Trace	Trace	—	—	—	Trace
Arachidonic	2.9	Trace	11.5	—	—	—	6.2
Poly-unsat. C ₂₀	Trace	Trace	2.5	—	—	—	5.0

Table 6. *Comparison of specific activities of fatty acids isolated from lipids of α- and β-lipoproteins*

—, Not determined, as quantity was too small; 0, zero value; m.u., mono-unsaturated. Results are in counts/min./mg.

Acid	α-Lipoprotein				β-Lipoprotein	
	Neutral fat		Phospholipid		Neutral fat + phospholipid Subject 1	Phospholipid Subject 2
	Subject 1	Subject 2	Subject 1	Subject 2		
<i>n</i> -C ₁₂	—	1800	—	—	—	—
m.u. C ₁₄	856	520	378	—	—	96
<i>n</i> -C ₁₄	6800	4600	515	—	437	520
m.u. C ₁₆	164	172	—	—	—	—
<i>n</i> -C ₁₆	1520	1045	30	124	81	87
Di- + tri-unsat. C ₁₈	54	123	0	0	—	—
m.u. C ₁₈	355	120	18	—	—	388
<i>n</i> -C ₁₈	900	1015	61.0	148	—	533
Arachidonic	322	—	20	—	—	88
Poly-unsat. C ₂₀	—	—	77.0	—	—	192

Table 7. *Specific activities of the components of the α -lipoprotein neutral fat separated on a silicic acid chromatogram*

Eluting solvent	Fraction no.	Percentage of total extract	Composition	Counts/min./mg. (uncorrected)
Ether-light petroleum (b.p. 40-60°) (1%, v/v)	1	47.4	Cholesterol esters + hydrocarbon	0.5
Ether-light petroleum (b.p. 40-60°) (4%, v/v)	2	17.1	Triglycerides + some free sterol	48
Ether-light petroleum (b.p. 40-60°) (10%, v/v)	3	7.3	Free sterol + unknown lipid	82
Ether	4	3.6	Unknown	52
Acetone	5	8.4	Unknown	19

Table 8. *Fatty acid composition of components of the neutral fat of α -lipoproteins*

0, Zero value; trace, less than 1%; m.u., mono-unsaturated; br., branched (*iso*- or *ante-iso*); h.br., highly branched. Composition is given as percentages.

Acid	Fraction no. (see Table 7)				
	1	2	3	4	5
<i>n</i> -C ₇	2.0	0	0	0	0
<i>n</i> -C ₈	0	0	9.6	5.1	Trace
br. C ₉	0	0	0	Trace	5.7
<i>n</i> -C ₉	0	0	6.0	6.1	10.9
<i>n</i> -C ₁₀	0	0.1	4.3	1.6	2.3
h.br. C ₁₁	0	0	0	1.8	0
br. C ₁₁	0	0	0	0	3.7
h.br. C ₁₂	0	0	0	2.0	4.1
m.u. C ₁₂	0	0.4	0	0	0
<i>n</i> -C ₁₂	Trace	2.2	37.0	9.7	29.3
h.br. C ₁₂	0	0	0	2.9	0
h.br. C ₁₄	0	0	0	6.4	0
<i>n</i> -C ₁₄	1.1	5.6	11.2	8.0	12.2
h.br. C ₁₅	0	0	0	10.0	0
br. C ₁₅	Trace	0.5	Trace	Trace	Trace
<i>n</i> -C ₁₅	Trace	0.92	Trace	Trace	Trace
h.br. C ₁₆	0	0	0	9.5	0
m.u. C ₁₆	3.6	4.5	0.5	0	0
<i>n</i> -C ₁₆	9.4	25.9	7.6	7.7	11.5
h.br. C ₁₇	0	0	0	7.3	0
h.br. C ₁₈	0	0	0	4.2	0
Di- + tri-unsat. C ₁₈	56.5	10.9	5.4	4.0	2.5
m.u. C ₁₈	22.0	40.5	11.0	8.4	13.0
<i>n</i> -C ₁₈	0	7.7	2.6	2.6	4.8

Table 9. *Specific activities of fatty acids isolated from components of the neutral fat of α -lipoprotein*

Results are in counts/min./mg. —, Not determined, as quantity was too small; h.br., highly branched; m.u., mono-unsaturated.

Acid	Fraction no. (see Table 7)				
	1	2	3	4	5
<i>n</i> -C ₁₂	No activity	—	27	—	4
<i>n</i> -C ₁₄		311	208	—	15
<i>n</i> -C ₁₆		181	94	21	10
h.br. C ₁₇		—	—	13.0	—
Di- + tri-unsat. C ₁₈		Low	Low	—	—
m.u. C ₁₈		4	7	—	Low
<i>n</i> -C ₁₈		62.0	34.0	40.0	—

Table 10. *Specific activities of components of the neutral fat of the erythrocyte*

Fraction no. (see Table 7)	Percentage of total extract	Composition	Counts/min./mg. (uncorrected)
1	25.2	Cholesterol esters + hydrocarbon	14
2	6.4	Triglycerides + some free sterol	549
3	61.3	Free sterol + unknown lipid	7.0
4	<1	Unknown	Labelled
5	3.8	Unknown	106

Table 11. *Specific activities of fatty acids isolated from components of the neutral fat of the erythrocyte*

—, Not determined, as quantity was too small; h.br., highly branched; m.u., mono-unsaturated. Results are given in counts/min./mg.

Acid	Fraction no. (see Table 7)			
	1	2	3	5
h.br. C ₁₂	No activity	46	—	—
n-C ₁₂		113	—	—
n-C ₁₄		595	—	32
m.u. C ₁₆		110	—	—
n-C ₁₈		352	11	6
Di-unsat. C ₁₈		51	—	—
m.u. C ₁₈		151	—	—
n-C ₁₈		700	20	—
Arachidonic		169	—	—
Poly-unsat. C ₂₀				

Table 12. *Comparative composition of neutral fat fractions from erythrocytes (see Table 10)*

—, Not determined, as quantity was too small; 0, zero value; trace, less than 1%; br., branched; h.br., highly branched; m.u., mono-unsaturated.

Acid	Fraction no. (see Table 7)			
	1 (%)	2 (%)	3 (%)	5 (%)
br. C ₆	0	0	High, not estimated	0
n-C ₆	0	0		0
br. C ₇	0	0		0
n-C ₇	0	0		0
br. C ₈	0	0		0
n-C ₈	0	0	1.8	0
br. C ₉	6.0	0	0	0
n-C ₉	0.2	3.2	21.9	12.5
br. or unsat. C ₁₀	0.6	0	Trace	1.1
n-C ₁₀	1.2	0.3	3.3	1.5
h.br. C ₁₁	0.8	0	Trace	Trace
br. C ₁₁	0.4	0	1.9	0.7
n-C ₁₁	1.2	0	0	4.4
h.br. C ₁₂	0.6	1.6	6.4	Trace
n-C ₁₂	1.7	2.7	14.2	2.5
br. C ₁₃	Trace	1.0	0	6.5
n-C ₁₄	2.3	6.1	20.2	4.6
h.br. C ₁₅	0	1.8	3.9	5.7
br. C ₁₅	Trace	0.3	Trace	Trace
n-C ₁₅	Trace	0.3	Trace	Trace
m.u. C ₁₆	2.2	3.2	0.9	1.8
n-C ₁₆	20.9	21.6	10.0	18.6
br. + unsat. C ₁₇	Trace	0.8	2.7	4.6
n-C ₁₇	Trace	0.4	Trace	Trace
Di-unsat. C ₁₈	25.4	11.1	3.3	3.4
m.u. C ₁₈	21.0	31.7	5.9	21.4
Isomers of oleic	2.2	3.7	Trace	Trace
n-C ₁₈	8.8	8.3	4.0	10.5
Arachidonic	4.8	1.2	—	—
Poly-unsat. C ₂₀	—	0.8	—	—

Marked differences in composition of the neutral fat and phospholipids of the two fractions can be seen; in particular the neutral fat of the α -lipoprotein contains less palmitic acid and stearic acid, but more oleic acid than the phospholipid.

Table 6 shows the specific activities of the isolated fatty acids. Very high activity is found only in the neutral fat of the α -lipoprotein, the descending order of activity being: α -lipoprotein neutral fat, β -lipoprotein phospholipid, α -lipoprotein phospholipid. The β -lipoprotein neutral fat showed very low activity and is not included as such in Table 6.

The fatty acids of the neutral fat of the α -lipoprotein complex arise from both cholesterol esters and from triglycerides. Insufficient material remained from the earlier experiments for further fractionation of the plasma lipids so a large-scale experiment was carried out with 100 ml. of blood. The fractionation was performed as before with the exception that the α -lipoprotein neutral fat was separated into five fractions by chromatography on silica gel. In Table 7 are summarized the properties of these fractions. The cholesterol esters show barely any activity and the triglycerides are highly labelled, as are the other three fractions of unknown constitution. Table 8 gives the fatty acid composition of these fractions. As expected, the cholesterol esters contain only 10% of saturated acids and are particularly rich in oleic acid and linoleic acid.

In Table 9 are given the specific activities of the acids isolated from all five fractions. No activity could be demonstrated in the acids from the cholesterol esters, but high activity was found in the acids from the triglycerides.

Not all the radioactivity of mixtures of fatty acid esters from these experiments was recoverable in acids in the range C_8 - C_{20} . The residual activity was found in material running more slowly through the columns and consisting presumably of C_{20} and C_{22} unsaturated acids, since these are known to be present in plasma lipids.

A similar separation was carried out on the neutral fat isolated from the erythrocytes of the large-scale experiment. In Table 10 are given the specific activities of the five fractions obtained from the silicic acid chromatogram. Again the highest activity is found in the triglyceride fraction; all other fractions did, however, show some degree of labelling. The specific activities of the fatty acids isolated from the fractions are shown in Table 11; the acids from the triglyceride fraction have higher activities than those found in the similar fraction from the α -lipoproteins. However, the fatty acid composition of this fraction is similar to that of the triglyceride fraction from the α -lipoprotein, whereas the cholesterol esters from these two lipids are quite different in composition (Table 12).

Tables 13 and 14 show the specific activities of the non-saponifiable materials isolated from the fractions of the α -lipoprotein and erythrocyte neutral fat. Some degree of labelling exists in all except fraction I, which consists of cholesterol esters. The free cholesterol forming the contaminant of fraction 2 is, however, labelled, supporting the view that the cells in human blood are unable to synthesize cholesterol esters but can synthesize cholesterol.

Composition of the neutral-fat fractions

The neutral fat from both erythrocytes and the α -lipoprotein was resolved into five fractions by means of a liquid-solid chromatogram.

Fraction 1. This fraction was eluted under conditions that define it as a mixture of cholesterol esters and hydrocarbons. The fatty acids and non-saponifiable material from both sources were unlabelled. This fraction accounted for 47% of the neutral fat in the α -lipoprotein and 25% in the erythrocyte.

The fatty acid composition of this cholesterol ester fraction from the α -lipoprotein was that expected (Table 8), being largely linoleic acid and oleic acid with only 9.4% of palmitic acid and no stearic acid whatsoever. The similar fraction from the erythrocyte possessed a quite different fatty acid composition (Table 12), with appreciable amounts of the saturated acids, suggesting little or no exchange of this material between erythrocytes and α -lipoprotein.

Fraction 2. This fraction (triglyceride) from both sources was appreciably labelled and contained a

Table 13. *Non-saponifiable material of fractions of α -lipoprotein neutral fat*

Fraction no. (see Table 7)	Percentage of non-saponifiable material	Specific activity (counts/min./mg.) (uncorrected)
1	64 (2.5 mg.)	0
2	< 5	Labelled
3	65 (3.9 mg.)	7
4	26 (0.8 mg.)	2
5	35 (2.4 mg.)	2

Table 14. *Specific activity of non-saponifiable material from fractions of erythrocyte neutral fat*

Fraction no. (see Table 7)	Percentage of total non-saponifiable material	Specific activity (counts/min./mg.) (uncorrected)
1	3 (0.5 mg.)	0
2	< 5	Labelled
3	67 (27.5 mg.)	3
4	?(< 0.1 mg.)	Labelled
5	< 10 (< 0.1 mg.)	0

small amount (less than 5%) of labelled non-saponifiable material (probably cholesterol) (Tables 13 and 14). The larger part of the activity, however, was due to the fatty acids, the composition being very similar in both cases (Tables 8 and 12); this suggests the concept of a rapid incorporation of the cell triglyceride into the α -lipoprotein. The fact that the individual fatty acids had higher specific activities in the erythrocyte than in the α -lipoprotein (Tables 9 and 11) agrees with the concept of a non-equilibrium state in the exchange process.

Fraction 3. This fraction contains a high proportion of labelled unsaponifiable material (about 65% in both α -lipoprotein and the erythrocyte). This fraction was the major component of the erythrocyte neutral fat (61%) but a minor constituent of the α -lipoprotein neutral fat (7.3%). In both cases the fatty acids were of unusual composition, very large amounts of the short-chain acids being present. Presumably most of this fraction is made up of uncombined sterol, and the saponifiable part would appear to be diglyceride, on the basis of its behaviour on the silicic acid chromatogram. If this is so then this diglyceride cannot be derived from either the cell triglyceride or the cellular phospholipid because of their different fatty acid compositions. The acids were labelled from both sources though their specific activities in the erythrocyte fraction were much lower than those in the α -lipoprotein fraction, unlike fraction 2. Here again is presumably an example of a non-equilibrium exchange.

Fraction 4. This was a minor component in both and contained some labelled non-saponifiable material; the fraction was so small in the erythrocyte that fatty acid determinations could not be carried out. The fraction from α -lipoprotein contained 26% of labelled non-saponifiable material and large amounts of the short-chain acids.

Fraction 5. This also was a minor constituent in both and the fatty acids were labelled, though much less than in other fractions. Traces of activity were found in the non-saponifiable material from the α -lipoprotein fraction.

The chemical nature of fractions 4 and 5 is unknown, but it is clear that part at least of these minor constituents of the α -lipoprotein and the erythrocyte are derived from the same source.

DISCUSSION

These experiments have demonstrated the ability of the cells in blood to take up acetate from the plasma and to synthesize a wide variety of saturated and unsaturated acids. In different experiments, between 2 and 4% of the acetate has been converted into long-chain fatty acids (cf. Altman,

1953), an appreciable amount when account is taken of the total weight of red and white cells in the body. [Acetate has been reported as a constituent of plasma by Fonnesu (1951).] The recovery of labelled acids from both neutral fat and phospholipids of the plasma shows that the cells also synthesize these lipids and secrete them into the plasma, where they are incorporated into the lipoproteins. Maximum activity is found in the triglycerides of the α -lipoprotein complex; next follow the phospholipids of the β -lipoprotein and the phospholipids of the α -lipoproteins; the β -lipoprotein neutral fat shows very little incorporation.

Incubation of red and white cells with labelled acetate in 0.9% sodium chloride instead of plasma showed that synthesis of fatty acids occurred as before, but little activity could be demonstrated in the 0.9% sodium chloride except for that due to acetate. The exchange of lipids with the fluid medium does not therefore occur rapidly unless the lipoproteins are present. The differential exchange with the α - and β -lipoproteins would suggest that in one case the neutral fat occupies the outer layer of the lipoprotein and in the other a less accessible inner space, the phospholipid here being more accessible.

Nature of the acids synthesized

The ability of the erythrocyte to synthesize fatty acids is not surprising, since Kaplan & Lipmann demonstrated in 1948 that human erythrocytes contain appreciable amounts of acetyl-coenzyme A. The amount reported (3–4 units/g. of wet tissue) is only about 3% of that found in rabbit liver. However, the total mass of erythrocytes is of the same order as that of the liver and their large surface area would allow ready exchange of synthesized lipids, not only with plasma lipoproteins but also with surfaces of blood vessels and capillaries. Although the results presented in this paper do not allow calculation of rates of synthesis, they do suggest that the red and the white cells, if they operate *in vivo* as they do *in vitro*, would contribute appreciable amounts of lipid to certain of the plasma lipoproteins. It is not possible at this stage to define the relative biosynthetic role of the reticulocytes, the erythrocytes and the various white cells. Studies are in progress to elucidate these points.

The high relative activity of the stearic acid appearing in the α -lipoprotein triglycerides suggests that the blood cells are one of the major sites of synthesis of this acid. In the unsaturated acids, the highest degree of labelling occurs in oleic acid and the acid described as 'poly-unsaturated C_{20} ' (having a retention volume relative to methyl stearate of 1.64). The incorporation of acetate into

this acid may be several times greater than that into arachidonic acid, suggesting that it may have some metabolic importance. The structure of this acid has been partially defined; on reduction, arachidic acid is formed and oxidative degradation has defined one of the double bonds at the 8:9 position and another at either the 14:15 or the 15:16 position. A study of the rate of movement of the intact acid on two types of gas-liquid chromatogram confirms the chain length as 20 carbon atoms and suggests that it possesses three double bonds.

The acid described as 'C₁₉A' has not been chemically defined in any way beyond demonstrating that it is unsaturated. It has a retention volume relative to methyl stearate of 1.27 under the chromatographic condition defined earlier.

The definite but relatively low radioactivity of the mixed peak of linoleic acid and linolenic acid is of interest since it is generally considered that these acids are 'essential' dietary constituents and are not synthesized in animal tissues. The presence of radioactivity in the molecule does not necessarily imply complete synthesis, since some exchange process is possible; e.g.

linoleate or
linolenate $\rightleftharpoons$ fragment + acetate $\rightleftharpoons$ acetate pool,

as well as a purely synthetic pathway such as

palmitoleate + acetate $\rightleftharpoons$ linoleate or linolenate.

The nature of the process of incorporation of acetate can only be defined by degradation of the molecule to determine the position of the radioactive atoms. So far the small amounts of material

obtained have prevented a complete degradation, but after oxidation labelled azelaic acid was successfully isolated.

Lipid exchange with the plasma lipoproteins

The specific activities of the fatty acids of the phospholipids of the erythrocyte and of both plasma-lipoprotein fractions are closely similar; this would suggest that the process of exchange between the erythrocyte and the plasma is more rapid than that of synthesis in the cell itself; this notion receives further support from the quantitative measurements of phospholipid exchange to be reported in a later paper. By contrast the specific activities of the various fatty acids of the triglycerides of the α -lipoprotein fraction are different from those observed with the cell triglycerides. This would suggest either a rate of synthesis by the red or white cells much more rapid than that of exchange with the plasma, or that the process of exchange with the plasma is irreversible. Quantitative measurements of the exchange process to be reported in a later paper indicate that the former suggestion is the more probable. The evidence so far accumulated is shown schematically in Fig. 1.

The evidence reported in this paper indicates that the cells in human blood have a remarkable capacity for lipid synthesis. In the wide variety of fatty acids isolated from the various lipid fractions all or almost all included isotopic carbon; this would suggest that human blood is a particularly convenient and suitable tissue for study of the various processes concerned in the metabolism of fatty acids.

Fig. 1. Schematic diagram of the lipid biosynthetic processes occurring in whole blood.

SUMMARY

1. The cellular components of human blood are shown to be a convenient system for studies of fatty acid and lipid synthesis and exchange.

2. After incubation of whole blood with [$Me-^{14}C$]acetate all the common saturated and unsaturated acids are found to be labelled, including the mono- and poly-unsaturated acids of a variety of chain lengths.

3. The 'essential' fatty acids arachidonic acid, and linoleic acid or linolenic acid or both, are also labelled.

4. The long-chain fatty acids are incorporated into triglycerides and some neutral lipids of undefined structure, as well as into phospholipids, but not into cholesterol esters.

5. The triglycerides and other neutral lipids are rapidly secreted into plasma, where they are incorporated into the plasma α -lipoproteins but not into β -lipoprotein.

6. The phospholipids are more readily incorporated into the plasma β -lipoproteins.

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REFERENCES

- Altman, K. I. (1953). *Arch. Biochem. Biophys.* **42**, 478.
 Altman, K. I. & Swisher, S. N. (1954). *Nature, Lond.*, **174**, 459.
 Altman, K. I., Watman, R. N. & Salomon, K. (1951). *Arch. Biochem. Biophys.* **33**, 168.
 Caffrey, R. W., Tremblay, R., Gabrio, B. W. & Huennekens, F. M. (1956). *J. biol. Chem.* **223**, 1.
 Fonnesu, A. (1951). *Bull. Soc. Chim. biol., Paris*, **33**, 1021.
 Hirsch, J. & Ahrens, E. H. (1958). *J. biol. Chem.* **233**, 311.
 James, A. T. & Lovelock, J. E. (1956). *The Blood Lipids and the Clearing Factor*. Brussels: Koninklijke Vlaamse Academie voor Wetenschappen.
 James, A. T., Lovelock, J. E. & Webb, J. (1957). *Biochem. J.* **66**, 60.
 James, A. T., Lovelock, J. E. & Webb, J. (1958). In *Essential Fatty Acids*, p. 72. Ed. by Sinclair, H. M. London: Butterworths Scientific Publications.
 James, A. T., Peeters, G. & Laurysens, M. (1956). *Biochem. J.* **64**, 726.
 James, A. T. & Webb, J. (1957). *Biochem. J.* **66**, 515.
 Kaplan, N. O. & Lipmann, F. (1948). *J. biol. Chem.* **174**, 37.
 Lovelock, J. E. (1955). *Biochem. J.* **60**, 692.
 Lowy, B. A., Ramot, B. & London, I. M. (1958). *Nature, Lond.*, **181**, 324.
 Muir, H. M., Perrone, J. C. & Popják, G. (1951). *Biochem. J.* **48**, 1.
 Oncley, J. L., Walton, K. W. & Cornwell, D. G. (1957). *J. Amer. chem. Soc.* **79**, 4666.
 Prankherd, T. A. J. (1955). *Brit. J. Haematol.* **1**, 131.

The Localization of ^{131}I in Biosynthetically Labelled [^{131}I]Thyroxine

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Work in this Laboratory on the biosynthesis and peripheral metabolism of thyroid hormones necessitated a method for the degradation of thyroxine whereby the proportions of radioiodine present on each of the two aromatic rings could be determined. The diazo-coupling reaction described in this paper was the best of several chemical reactions investigated with a view to displacing iodine specifically from the ring distal to the alanine side chain. Thyroxine, a phenolic coupling component, has no unsubstituted position *ortho* or *para* to the hydroxyl group. On coupling therefore iodine must be displaced from organic combination or the diphenyl-ether linkage is destroyed. The reaction has been studied by other workers, but never in conjunction with isotopic-tracer techniques. Komant (1950) coupled thyroxine with diazotized 2:4-dichloro-aniline. Barac & Morren (1953) studied the reaction

with sulphanilic acid and *p*-arsanilic acid. They isolated the *p*-arsanilic acid azo-compound in 60% yield and claimed that it was an azo-substituted tri-iodothyronine. It was then apparent that a large proportion of the diazo-coupling occurs by the elimination and replacement of the *ortho* substituent.

At best therefore in a reaction between equimolecular quantities of thyroxine and diazonium salt, where coupling is confined to the *ortho* position, a product of structure (I) is to be expected, where R is a substituted aryl nucleus. If the structural formula is correct, it follows that when [$3:5-^{131}I_2$]-thyroxine is subjected to diazo-coupling, no radioiodine is displaced from organic combination. If the ^{131}I were present on both rings in the same amounts, 25% would be converted into inorganic iodide. On theoretical grounds therefore it is