Incorporation of Acetate and Fatty Acids into Lipids of Rat Platelets[1] (35377)

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The ability of human platelets to incorporate *in vitro* acetate and fatty acids into their lipids was recently demonstrated by several investigators (1–4). Furthermore, platelets were shown to be the only formed elements of the human blood capable of *de novo* synthesis of fatty acids (5) and were also found to synthesize phospholipids (6). Different fatty acids exhibited distinctive patterns of incorporation into the various lipid classes of human platelets. Considerable variability of these results was observed depending upon the experimental methods used. With the red cells which are only capable of exchange of lipids with the medium, not of *de novo* synthesis, marked species differences were found in their ability to incorporate fatty acids into phosphoglycerides (7). Such comparative studies, however, have not yet been done on platelets. In the present research, using virtually pure platelet suspensions, we have examined the incorporation of acetate and of various fatty acids into different lipid classes of rat platelets and compared the results in these rodents with those reported by other investigators on human platelets.

Materials and Methods. Male Sprague-Dawley rats (280–350 g) were given water and Purina laboratory chow *ad libitum*. After overnight starvation, blood was collected with acid-citrate dextrose as anticoagulant; and platelets were separated by differential centrifugation (8). The platelets were washed twice with Gaintner's buffer (9), of pH 6.8, modified to contain no EDTA. Washed platelets from at least four rats were pooled and suspended in modified Gaintner's buffer of pH 7.4 (1×10^9 platelets/ml). Red and white cell contamination was always less than 1 per 25,000 and 1 per 100,000 platelets, respectively. Siliconized glassware was used throughout this procedure.

Palmitic, oleic, linoleic, and linolenic acid-1-^{14}C (Amersham-Searle; Des Plaines, Ill.) and acetate-1-^{14}C (New England Nuclear, Boston, Mass.) were mixed with the respective nonradioactive substance (fatty acids of $<99\%$ purity were obtained from Mann Research Laboratories, New York, N.Y.) to obtain the desired specific activity. Fatty acids were bound to 5% bovine serum albumin (BSA, crystallized; Mann Research Laboratories) in 0.1 *M* Tris–HCl buffer, pH 7.4. The fatty acids dissolved in benzene were evaporated almost to dryness under a stream of nitrogen, then added to the BSA solution and vigorously agitated on a Vortex mixer for 5 min. Subsequently the remaining benzene was driven off under a stream of nitrogen. In case this solution was not used promptly, it was divided into 0.2-ml aliquots, sealed in ampules under nitrogen and stored in the dark at —20° until used (up to 1 month). During this time, virtually no degradation of the fatty acids in such solutions occurred as shown by gas–liquid radio-chromatography (3, 10) (the purity of the fatty acids-1-^{14}C exceeded 95%).

Two ml aliquots of the platelet suspension were incubated at 37° in a metabolic shaking waterbath under an atmosphere of 95% O_2 and 5% CO_2 either with 0.2 ml of aqueous acetate-1-^{14}C, usually 10 μCi, or with 0.2 ml of albumin-bound fatty acid-1-^{14}C, usually 5 μCi. Platelets incubated with acetate were washed twice at 4° with 3 ml aliquots of ice-cold modified Gaintner's buffer of pH 6.8; whereas those incubated with fatty acids

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were subjected to five washing with 3 ml aliquots of ice-cold 0.5% BSA dissolved in the same buffer. Following the washing procedure the platelet lipids were extracted by the modified Folch procedure (3) and washed 3 times (11). The extract was evaporated under nitrogen and dissolved in a small volume of extraction solvent. Two small aliquots of this solution were set aside for determination of total radioactivity and PL[2] phosphorus (12) and the remainder was subjected to thin-layer chromatographic separation. Silica gel N-HR (Brinkman Instr., Westbury, N.Y.) was always used as the coating material. The major PL, *i.e.*, SM, PC, PI, PS and PE (Mann Research Laboratories) as well as LPC (Analab; North Haven, Conn.) were used as external reference standards and were resolved by chloroform–methanol–acetic acid–water (75:25:6:2, by vol) (3) (TLC I). The major lipid classes were separated using 2 developing systems according to Skipski *et al.* (13) (TLC II). PL, CEM, and CEB remained at or near the point of application but all the other lipid classes with the exception of CH and DG were clearly separated. With this system monopalmitin, diolein, palmitic acid, tripalmitin, arachidic acid methyl ester, and cholesteryl palmitate (Mann Research Laboratories) were always used as internal reference standards. CH and DG were separated by the method of Freeman and West (14) (TLC III). CEM, CEB, CL, and PA were resolved by the combined use of 2 developing systems: acetone–petroleum ether (1:3, by vol) and chloroform–methanol–acetic acid–water (80:13:8:0.3, by vol) (15) (TLC IV). CEM and CL (Applied Science Laboratories; State College, Pa.) as well as CEB and PA (Mann Research Laboratories) were used as internal reference standards. BHT (4-methyl-2,6-di-tert-butylphenol, Sigma Chemical Co.; St. Louis, Mo.) was added to all developing solvents in a concentration of 50 mg/100 ml.

For determination of radioactivity, the spots on the TLC plates corresponding to the individual lipids were scraped into scintillation vials, suspended in 5% (wt/vol) Cab-O-Sil (Cabot Co.; Boston, Mass) in toluene containing 0.4% (wt/vol) PPO and 0.05% (wt/vol) POPOP and counted in a liquid scintillation spectrometer. Aliquots of the original lipid extract were counted in the above scintillation system without Cab-O-Sil. Quenching characteristics were determined by using an internal standard of toluene-^{14}C.

Phosphorus was determined on each clearly identified major PL and on an area corresponding to LPC (12). Corrections were made for the absorbancy of corresponding areas of blank lanes.

Between 93 and 100% of the radioactivity and of the PL phosphorus spotted onto the TLC plates was recovered after chromatographic separation.

Results. Optimal conditions for incorporation of acetate and fatty acids into platelet lipids were established in preliminary experiments. Acetate and palmitate incorporation into lipids was linear up to 120 min. Changes in pH of the incubation medium from 6.8 to 7.4 did not affect the rate of incorporation of these substances into lipids. Based on these results all subsequent experiments were performed using 2×10^9 platelets suspended in the buffer (pH 7.4) for an incubation time of 60 min.

The rate of acetate incorporation increased linearly with its concentration in the medium up to 0.11 m*M*. At higher concentrations the rate was markedly less but up to 2.2 m*M* no definite saturation plateau was reached. The total incorporation into lipids was 0.1 nmoles/10^9 platelets/60 min at 0.11 m*M* and 0.15 nmoles/10^9 platelets/60 min at 2.2 m*M* acetate in the medium. In order to obtain samples of sufficiently high count rate 0.11 m*M* acetate (sp act, 40 mCi/mmole) was used in all subsequent experiments. The acetate incorporated into lipids constituted about 23% of the total uptake of acetate by

[2] The following abbreviations are used in the text and illustrations: TLC, thin-layer chromatography; FFA, free fatty acids; PL, phospholipids; SM, Sphingomyelins; PC, phosphatidylcholines; PI, phosphatidylinositols; PS, phoshatidylserines; PE, phosphatidylethanolamines; LPC, lysophosphatidylcholines; MG, monoglyerides; DG, diglycerides; TG, triglycerides; CH, cholesterol; CE, cholesteryl esters; FAE, fatty acid methyl esters; CL, cardiolipin; PA, phosphatidic acids; CEM, ceramides; CEB, cerebrosides; PPO, 2, 5-diphenyloxazole; POPOP, *p*-bis [2-(5-phenyloxazolyl)]-benzene.

TABLE I. *In Vitro* Incorporation of Acetate-1-^{14}C and Fatty Acids-1-^{14}C into Rat Platelet Lipids and Distribution of Radioactivity Among the Lipid Classes.

	Acetate	Palmitate	Oleate	Linoleate	Linolenate
Total incorporation (nmole/10^9 platelets)	0.08 ± 0.01[a]	4.2 ± 0.2	2.0 ± 0.2	2.0 ± 0.1	2.0 ± 0.2
			Distribution (%)		
LPC	tr[b]	tr	tr	tr	tr
SM	2.6 ± 0.3	2.5 ± 0.3	1.6 ± 0.1	2.0 ± 0.2	2.0 ± 0.2
PC	20.5 ± 0.6	34.4 ± 0.8	39.8 ± 1.5	49.2 ± 1.0	42.8 ± 1.4
PI + PS	7.0 ± 0.5	1.4 ± 0.1	5.5 ± 0.5	3.7 ± 0.4	3.2 ± 0.6
PE	10.9 ± 0.7	6.7 ± 0.5	14.8 ± 0.7	13.9 ± 0.3	14.4 ± 0.4
CL	tr	tr	tr	tr	tr
PA	tr	tr	tr	tr	tr
CEM	15.4 ± 0.7	2.3 ± 0.4	1.7 ± 0.3	1.8 ± 0.4	6.6 ± 0.5
CEB	tr	tr	tr	tr	tr
MG	5.3 ± 0.4	tr	tr	tr	tr
CH	2.2 ± 0.3	tr	tr	tr	tr
DG	5.5 ± 0.2	5.5 ± 0.5	3.2 ± 0.3	3.1 ± 0.3	10.5 ± 0.8
FFA	13.1 ± 0.5	9.1 ± 1.0	6.5 ± 0.6	6.8 ± 0.3	8.0 ± 0.4
TG	15.6 ± 1.6	36.7 ± 1.0	25.5 ± 4.4	17.4 ± 0.5	9.9 ± 0.7
FAE	tr	tr	tr	tr	tr
CE	tr	tr	tr	tr	tr

[a] Values represent mean ± SEM of 4 expts.
[b] Trace: less than 0.9%.

platelets. With palmitate 0.22 m*M* (sp act, 10 mCi/mmole) was found to be a saturating concentration, which was then used in all further incubations.

The total incorporation of acetate and various fatty acids into platelet lipids and the distribution of radioactivity among the various lipid classes is shown in Table I. The incorporation of palmitate was highest, 4 nmoles/10^9 platelets/60 min, about twice that of any of the other fatty acids. *De novo* synthesis of fatty acids by comparison was much lower, only 0.08 nmoles acetate were incorporated into the lipids of 1 × 10^9 platelets/60 min.

Since the platelet suspensions utilized in these experiments were always contaminated with some red and white cells it appeared of importance to determine the contribution of such blood cells to the observed acetate and fatty acid incorporation into lipids. Rat erythrocytes were found unable to incorporate acetate-^{14}C into red cell lipids. To assess the significance of the contribution of leukocytes to the synthetic activity of platelets the standard system of incubation was modified to contain variable numbers of white cells while holding the platelet concentation constant. There was no significant change in the incorporation of acetate into lipids when white cell contamination was increased up to 7 times its maximal level usually encountered in the platelet suspensions (Table II). Similarly the incorporation of palmitate-1-^{14}C was not significantly affected by red and white cell contamination up to 7 times their usual level (Table II). Based on these findings we feel that neither leukocytes nor red cells in the platelet suspensions contributed significantly to the observed acetate and fatty acid incorporation.

Approximately 40% of the total acetate radioactivity incorporated was found in PL among which PC and PE showed the main uptake (Table I). Among neutral lipids, TG, CEM, and FFA had similar rates of incorporation which were much greater than those of MG and DG. There was clear evidence for CH synthesis although of very limited extent. Each of the fatty acids used showed a distinctive pattern of incorporation into platelet lipids. PC, PE, and TG constituted

TABLE II. Significance of Red and White Cell Contamination of Platelet Suspensions for Acetate and Palmitate Incorporation into Lipids.

Platelets/flask	Leukocytes/flask	Acetate incorporation into lipids (nmoles/60 min)
2×10^9	8.5×10^3	0.15
2×10^9	29.0×10^3	0.14
2×10^9	55.0×10^3	0.15
2×10^9	70.0×10^3	0.15

		Erythrocytes/flask	Palmitate incorporation into lipids (nmoles/60 min)
2×10^9	$<5 \times 10^2$	$<5 \times 10^3$	8.6
2×10^9	18×10^3	18×10^4	8.6
2×10^9	70×10^3	70×10^4	8.8

the principal groups of lipids which utilized fatty acids from the medium and TG exhibited the greatest absolute and relative difference of incorporation among the various fatty acids. The greater the degree of unsaturation of the fatty acids, the lower their utilization in TG. Incorporation either of acetate or of fatty acids into PA, CL, CEB, LPC, FAE, and CE was negligible. The low activity observed by direct counts of these individual spots could also be confirmed indirectly for PA, CL, LPC, and CEB by showing that the difference between radioactivity of the combined major PL classes as separated by TLC I and that of the origin of TLC II containing major PL, LPC, CEM, CEB, CL, and PA corresponded to the radioactivity found in the CEM spot separated by TLC IV.

The specific activities of the major PL classes are shown in Figs. 1 and 2 for platelets incubated with acetate and fatty acids, respectively. When *de novo* synthesis of fatty acids was studied, PI + PS had the highest turnover together with PC ($p<0.2$) and PE ($p<0.1$), followed at a significantly lower level by SM ($p<0.001$). The incorporation of fatty acids into PL showed 50–100 times higher specific activities than were found with acetate.

The various fatty acids displayed certain characteristic differences in their respective specific activities which were most marked when comparing saturated with unsaturated fatty acids. Although all the fatty acids studied had their greatest turnover in PC, palmitate exceeded the unsaturated acids two- to threefold in its turnover rate in SM. It more or less equaled oleate, linoleate, and

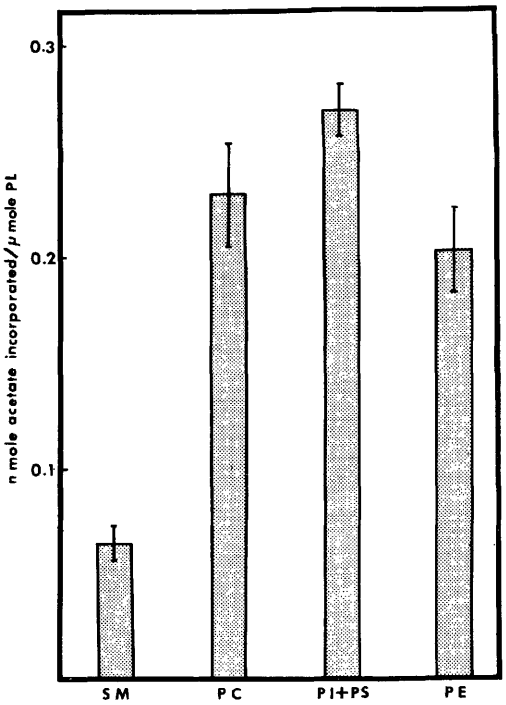

FIG. 1. *In vitro* incorporation of acetate-1-^{14}C into major PL of rat platelets. Two billion washed rat platelets were suspended in 2.0 ml of buffer (pH 7.4) and incubated with 0.25 μmole of Na acetate-1-^{14}C at 37° for 1 hr under 95% O_2 and 5% CO_2. Each bar represents the mean ± SEM of 4 expts.

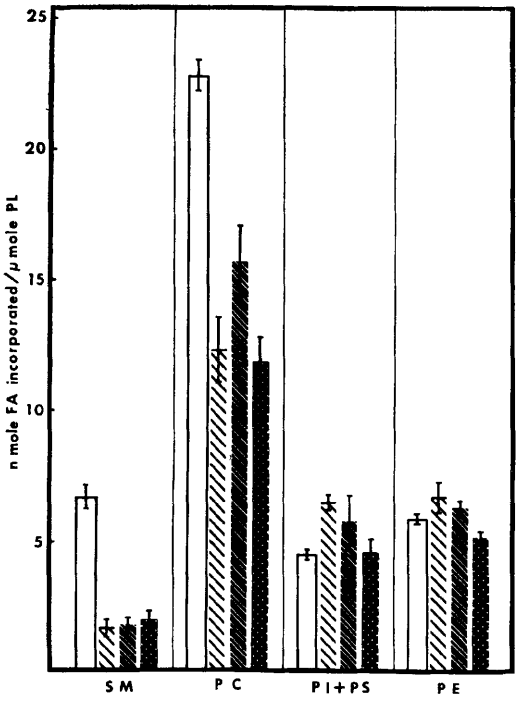

FIG. 2. *In vitro* incorporation of palmitate-1-^{14}C, oleate-1-^{14}C, linoleate-1-^{14}C, and linolenate-1-^{14}C into major PL of rat platelets. 0.5 μmole of FA-1-^{14}C bound to BSA was used in the incubation mixture. Other experimental conditions were identical to those described in legend to Fig. 1. Each bar represents the mean ± SEM of 4 experiments; (open bar) palmitate; (single stripe) oleate; (double stripe) linoleate; (dotted bar) linolenate.

linolenate in PI + PS and PE. Significant differences among the unsaturated fatty acids were found in PC in which linoleate had a higher specific activity than either oleate ($p<0.05$) or linolenate ($p<0.02$) and in PI + PS in which oleate was highest being significantly different from palmitate ($p<0.02$) and from linolenate ($p<0.02$).

Discussion. These experiments demonstrate that rat platelets are capable of *de novo* synthesis of fatty acids and that certain lipid constitutents of rat platelets are renewed by incorporation of nonesterfied fatty acids from the surrounding medium. The maximum rate of acetate incorporation into human platelet lipids was reported to be 0.6 to 0.8 nmoles/10^9 platelets/hr (1, 3) and thus exceeds by far the maximal synthetic rate in rat platelets which we found to be 0.15 nmoles/10^9 platelets/hr. A species difference was also found in the relative distribution of acetate among the various lipid classes. While human platelets showed the highest incorporation of acetate into CER, followed by PC and FFA (3), in rat platelets PC was found to have the greatest rate of synthesis followed by TG and CER. Rat platelets were able to synthesize CH similarly to human platelets (3). The distribution of palmitate among the different lipids showed also a striking difference when comparing human (3) with rat platelets. In these, the relative incorporations into TG and FFA were greater and those into PC, PI + PS and CER were smaller than in human platelets. Since the concentrations of TG and DG in rat platelets are much lower than those of PL,[3] it is very probable that the specific activities of the neutral lipids with respect to fatty acid incorporation, particularly of TG, are much higher than those of PL. Substantial amounts of fatty acids were found incorporated into DG which is probable evidence for a high turnover of acyl residues in neutral lipids in rat platelets.

Lipids of rat platelets appear to be in a dynamic state, capable of considerable reacylation by incorporation of fatty acids from the medium and exhibiting a limited ability of *de novo* synthesis of fatty acids. Compared with data from human platelets (3), differences existed in the rate of *de novo* synthesis of fatty acids and in their incorporation characteristics with respect to lipid classes and type of fatty acids utilized.

Summary. The incorporation of acetate and fatty acids into rat platelet lipids was studied by incubating washed platelets with acetate-1-^{14}C or albumin-bound fatty acids-1-^{14}C (palmitic, oleic, linoleic, and linolenic acid) in an artificial medium without addi-

[3] When lipids extracted from 0.4 × 10^9 platelets were applied to TLC II, spots of PL and CH were clearly visible after spraying with Rhodamine 6G. However, no other neutral lipids were detected without their internal references. This indicated that concentrations of PL and CH are much higher than other neutral lipids, although no quantitative analysis was performed.

tion of cofactors. Acetate was incorporated primarily into PC, TG, CEM, FFA, and PE which accounted for three-fourths of its total incorporation into lipids. Palmitate incorporation into platelet lipids was twice as high as that of each of the unsaturated fatty acids. A distinctive pattern of distribution of palmitate or of the unsaturated fatty acids among the various lipid classes was observed as well as differences in the relative abundance of the fatty acids incorporated into each PL group. Rat platelets are therefore capable of (i) incorporation of fatty acids from the suspending medium, and (ii) *de novo* synthesis of fatty acids. Pattern of fatty acid incorporation and rate of *de novo* synthesis appear to be different from those in human platelets.

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