Transient thrombopenia after intravenous injection of certain fatty acids

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SUMMARY *Intravenous injections of various fatty acids in* rabbits caused marked thrombopenia lasting 1–2 hr. The most active saturated fatty acids were myristic acid (14:0) and lauric acid (12:0). Activity decreased with increasing and decreasing chain length, but behenic acid (22:0) had, on a molar basis, activity similar to that of lauric acid. Of the unsaturated fatty acids, oleic acid (18:1) was active only at high doses, whereas linoleic (18:2) and linolenic acid (18:3) had an effect comparable to palmitic acid (16:0). Intraperitoneal administration of the fatty acids caused no thrombopenia. The thrombopenic effect of the fatty acids was greatly reduced if the solutions were mixed with serum before injection.

Hyperlipemia is thought to be an important factor in the pathogenesis of thrombosis (1) since it is associated with increased adhesiveness of the thrombocytes (2) and hypercoagulability of the blood (3, 4). Recent experimental findings suggest that some fatty acids also enhance blood coagulation and thrombocyte aggregation: the clotting time of citrated plasma was shortened after addition of certain fatty acids (5–7). Formation of an artificial platelet thrombus can be observed when citrated human blood is rotated in a circular tube (8). After addition of various fatty acids to the blood in the proportion of 0.1%, thrombus formation was markedly accelerated (9, 10). In these experiments, the long-chain saturated fatty acids had the most pronounced effect and the activity diminished with decreasing chain length, caproic acid (6:0) being inactive. None of the unsaturated fatty acids tested had any effect on the in vitro thrombus formation. Similarly, it was found by Shore and Alpers (11) that addition of fatty acids in amounts as small as 1–5 μg/ml to platelet-rich rabbit plasma caused platelet clumping and platelet damage characterized by marked release of serotonin and histamine. In this experimental model, the long-chain saturated fatty acids behenic, arachidic, and palmitic acids were highly active, whereas little activity was seen with the shorter chain saturated fatty acids or with oleic acid.

Since all these effects of fatty acids on platelets had been observed in in vitro systems, it seemed interesting to investigate what would happen to the blood platelets if fatty acids were administered to intact animals. For such experiments, the poor solubility of the fatty acids at pH 7.4 presents the most important difficulty. Injectable forms of various fatty acids suitable for intravenous administration were prepared for this study by raising the pH of the solutions above 8.0. It is for this reason that the data presented in this paper must be viewed essentially as results of model experiments that may not be directly compared with the possible effects of endogenously produced fatty acids.

METHODS

New Zealand albino rabbits of both sexes, weighing between 1.7 and 3.2 kg, were used in all experiments. The composition and pH of the injectable solutions and the sources and purity of various fatty acids are listed in Table 1. Pyrogen-free water was used and the pH was adjusted with NaOH. Benzyl alcohol was added as preservative. For control experiments, injectable solutions of pyrogen-free water with the pH adjusted to 10.0 and an acetate solution containing sodium acetate equivalent to 5 mg/ml of acetic acid, also adjusted to pH 10.0, were prepared. Since the purity of our first samples of stearic, palmitic, lauric, and oleic acid was rather low, some of the experiments were repeated with fatty acids of more than 99% purity. Three preparations had to be warmed before injection. The temperature at the time of injection was 38–39° for palmitic, 37–40° for stearic, and 60° for behenic acid.

The fatty acid solutions were injected slowly (30 sec) intravenously in the ear veins. The thrombocyte counts were determined immediately before injection and 5, 10,

60, and 120 min thereafter in the free-flowing blood obtained by an incision of an ear vein. Every thrombocyte count was done independently by two, or occasionally three, trained technicians using a direct counting method described by Klein (12). If the determinations differed significantly, the results were discarded. The precision of the technicians was checked by comparison of repeated thrombocyte counts of normal rabbits. In rabbit 61, technicians C and V counted the thrombocytes five times. The coefficient of variation was 6.1% for C, 7.5% for V, and 6.7% for the pooled results of both. Three technicians determined the thrombocyte counts of rabbit 58 five times. In this case the coefficient of variation was 5.5% for C, 12.1% for V, 9.2% for N, and 9.3% for the pooled data. Consequently, approximately two-thirds of the observations from an experiment with the same precision as these data from rabbits 61 and 58 will lie 6.7% and 9.3%, respectively, above or below the true mean. Variations of the thrombocyte counts of 20% of the initial values or less were therefore excluded from further evaluation.

In a few experiments, lauric acid was also administered intraperitoneally. Furthermore, the lauric acid solution was mixed with varying amounts of rabbit serum before intravenous injection. Of the 93 rabbits included in this study, 36 were used for one, 52 for two, and 5 for three experiments. Between experiments, all animals had a rest period of at least 1 week. Under these conditions, thrombocyte changes did not vary significantly after the first, second, and third administration of fatty acids, as demonstrated in an example in Table 2. In order to determine whether or not the intravenous injections of fatty acids caused marked platelet destruction the excretion of 5-hydroxyindole acetic acid (5-HIAA) in the urine was measured in eight rabbits before and after a single administration of 5 mg/kg of myristic or lauric acid. The 5-HIAA was determined by the spectrophotofluorimetric method of Udenfriend et al. (13).

RESULTS

Tolerance of the Fatty Acid Injections

In the majority of the cases the intravenous injection of various fatty acids caused no adverse reactions, with the exception of two animals which died 2 min after intravenous injection of 2 mg/kg of behenic acid. The cause of death was not determined, but may be related to the high temperature and the large volume of the injected solutions. After termination of the experiments, the rabbits were observed for several weeks. Four died spontaneously. All had been treated with myristic acid in doses ranging from 1 mg/kg to 5 mg/kg. The time interval between treatment and death was between 10 and 21 days. The remaining animals showed no adverse aftereffects. At autopsy no signs of extensive thrombosis were observed.

Effect on Thrombocyte Count

In normal rabbits, the thrombocyte counts vary considerably from one animal to another. In a series of 100 animals, the initial counts were found to be between 220,000 and 784,000 per mm^3, the mean being 459,000 ± 96,000. For individual rabbits, however, the thrombocyte counts remained fairly stable, as seen in the examples of Table 2.

As controls, 10 rabbits received 1 ml/kg or 2 ml/kg of the diluent intravenously. Only minor changes were recorded and the thrombocyte count never fell below 93% of the initial values. In two animals, a moderate increase of the thrombocyte counts was noted. The average count, 5 min after diluent injection, was 101 ± 6% of the initial values (Table 1). In contrast, the intravenous injection of fatty acids was often followed by a rapid decrease in the number of circulating thrombocytes, which in extreme cases amounted to more than 80% of the initial values. The thrombocyte counts generally returned to, or near to, the initial values after 1–2 hr.

The largest series of experiments was performed with lauric acid. It is summarized in Table 1, which shows the percentage decrease of the blood platelet counts in 15 rabbits treated with various doses of lauric acid. With 5 mg/kg a decrease of more than 60% in thrombocyte counts was produced. With 2.5 mg/kg four out of six rabbits showed a reduction of more than 50% in the platelet counts. Lower doses produced less pronounced changes, with the exception of one animal, treated with 0.62 mg/kg, which exhibited a marked drop in the thrombocyte count. Table 1 also contains the data obtained with other fatty acids. It is evident that 14:0 and 12:0 had the most pronounced effect on platelet counts and showed activity at dosages as low as 0.6 mg/kg. Only a slight drop in the thrombocyte count occurred after injection of 10:0, 8:0, and 6:0, even at doses as high as 10 mg/kg. These changes are not considered of practical significance. Sodium acetate at 5 mg/kg and 10 mg/kg had no effect.

Activity also decreased as chain length increased, 16:0 being essentially inactive at 1.25 mg/kg and 18:0 showing activity only at 5 mg/kg. Behenic acid (22:0), however, evinced modest activity at 1.0 mg/kg and was therefore, on a molar basis, nearly as active as lauric or myristic acid. Among the unsaturated fatty acids, 18:3 was markedly active at 2.5 mg/kg, but essentially inactive at half this dose. Oleic acid (18:1) showed high and consistent activity only at 10 mg/kg and 18:2 was active at 5 mg/kg.

TABLE 1 EFFECT OF VARIOUS FATTY ACIDS ON PLATELET COUNTS IN RABBITS

Fatty Acid		Purity	Concentration	pH	Dose	Thrombocyte Count $\times 10^{-3}$ Initially	Minutes after Injection 5	10	60	120
			mg/ml		*mg/kg i.v.*	*per mm³*		*% of initial count*		
Behenic acid	(22:0)	>99%*	1	11.0	2	272	59	59	84	82
					2	489	70	75	99	—
					2	443	48	52	63	92
					1	455	68	79	98	—
					1	434	76	89	99	101
					0.5	386	100	107	117	—
					0.5	452	87	93	103	—
Stearic acid, Sample I	(18:0)	97%†	5	10.0	5	382	52	70	99	—
					5	397	55	72	100	105
					2.5	418	92	65	100	—
					2.5	477	91	91	102	—
					2.5	505	86	97	95	—
Palmitic acid, Sample I	(16:0)	90%‡	5	10.0	10	432	50	70	89	—
					10	450	47	79	70	73
					5	500	33	24	49	—
					5	220§	26	35	120	105
					2.5	475	71	83	108	105
					2.5	273§	12	35	82	94
					2.5	310	68	90	107	109
					1.25	384	95	88	95	107
					1.25	414	95	104	106	—
Myristic acid	(14:0)	>98%‖	5	10.0	5	444	9	11	68	92
					5	405	16	36	90	101
					5	311	8	21	76	90
					2.5	375	16	27	62	95
					2.5	545	26	66	75	94
					1	565	80	106	118	102
					1	333	67	85	91	100
					1	490	14	42	93	—
					0.6	488	61	69	90	97
					0.6	297	73	89	95	106
					0.6	494	63	80	89	100
Lauric acid, Sample I	(12:0)	¶	5	8.5–9.0	5	376	38	53	81	92
					5	361	31	30	85	92
					2.5	484	43	67	85	—
					2.5	448	66	82	83	99
					2.5	433	24	58	88	96
					2.5	459	58	74	88	114
					2.5	464	22	—	—	—
					2.5	501	25	—	—	—
					1.25	507	—	59	89	80
					1.25	365	64	69	107	—
					0.6	595	74	92	93	101
					0.6	537	36	52	60	—
					0.5	573	87	91	94	—
					0.3	237	91	89	129	—
					0.3	714	76	98	100	—
Capric acid	(10:0)	>98%**	5	9.2–9.5	10	477	91	100	103	—
					10	419	92	98	100	—
					10	496	85	114	77	—
					5	259	91	91	105	—

* Hormel Foundation, Austin, Minn.
† Humko Prod., Div. Ntl. Dairy Prod. Corp., Memphis, Tenn.
‡ Armour & Co., Chicago, Ill.
§ Same rabbit with low initial thrombocyte count.
‖ Foremost Food & Chem Co., Oakland, Cal.
¶ ("Highest Purity" mp 42–43°) Matheson-Coleman & Bell, Div. Matheson Co., E. Rutherford, N.J.
** Drew Chemical Corp., New York, N.Y.

Repetition of the experiments with stearic, palmitic, lauric, and oleic acids of high purity (Table 3) gave results similar to those obtained with the less pure material. Thus, the impurities are not responsible for the effect on the thrombocytes. Tables 1 and 3 also indicate a satisfactory dose–activity relationship in most cases. Determination of the 5-HIAA excretion in the urine before and 2 hr after intravenous injection of 5 mg/kg of lauric and myristic acid was performed in eight rabbits. In seven rabbits the urine collected after the fatty acid injection contained less, in one animal slightly more, 5-HIAA per milliliter than the urine obtained before the injections (Table 4).

In order to investigate the response of thrombocytes to repeated fatty acid injections, three rabbits were treated at short intervals with 2.5 mg/kg of lauric acid. As demonstrated in Table 2, the drop in thrombocyte count was of the same order of magnitude after the first, fourth, and sixth injection and quite different from the response of the animals in a previous experiment with different fatty acids (rabbits 66 and 72) or a much lower dose of lauric acid (rabbit 74).

When the injectable preparations of fatty acids were brought in contact with serum in vitro, a precipitate (containing 60–70% lipids, 30–40% water-soluble material) was formed. A mixture containing 3 parts of the

TABLE 1 (*Concluded*)

Fatty Acid		Purity	Concentration	pH	Dose	Thrombocyte Count $\times 10^{-3}$ Initially	Minutes after Injection 5	10	60	120
			mg/ml		*mg/kg i.v.*	*per mm³*		*% of inital count*		
Caprylic acid	(8:0)	>98%**	5	9.2–9.5	10	360	79	82	100	—
					10	363	89	98	101	—
					10	456	98	103	105	—
					5	351	97	95	100	94
Caproic acid	(6:0)	>98%**	5	9.2–9.5	10	343	89	92	101	—
					10	387	89	96	101	—
					10	530	91	95	90	—
					5	514	94	93	97	—
Acetic acid	(2:0)	100%	5	10.0	10	544	100	105	87	—
					10	327	101	100	98	89
					5	495	100	102	93	—
					5	540	93	95	89	—
Oleic acid, Sample I	(18:1)	ca. 70%††	5	10.0	10	352	32	59	51	—
					10	409	16	20	54	—
					5	385	97	104	117	107
					5	521	—	84	96	96
					5	463	98	98	100	—
Linoleic acid	(18:2)	>99%*	5	9.5–10.0	5	588	45	73	82	98
					5	364	26	33	76	79
					2.5	518	72	88	103	—
					2.5	386	49	76	78	105
					2.5	385	104	100	97	—
					1.25	724	92	93	95	99
					1.25	348	88	94	103	—
Linolenic acid	(18:3)	>99%*	5	8.5	5	451	27	40	72	102
					5	377	52	77	98	102
					2.5	452	46	54	91	100
					2.5	599	22	48	88	90
					1.25	532	98	100	101	—
					1.25	354	91	91	106	—
Diluent		—			1 ml/kg	535	104	103	—	—
					1 "	370	99	136	—	—
					1 "	426	93	100	101	—
					1 "	370	95	106	95	—
					1 "	338	101	101	103	—
					2 "	313	113	132	137	139
					2 "	411	104	109	101	97
					2 "	436	95	100	94	—
					2 "	434	102	100	98	—
					2 "	318	104	104	100	—

†† U.S.P. 1% Benzyl alcohol was added to all solutions as preservative.

TABLE 2 THROMBOCYTE COUNTS AFTER REPEATED INTRAVENOUS INJECTIONS OF FATTY ACID SOLUTIONS

Rabbit No.	Day	Fatty acid	Dose	Thrombocyte Counts × 10^{-3} Initially	Thrombocyte Counts 5 Min after Injection
			mg/kg	*per mm³*	*% of initial*
66	0	Linolenic acid	1.25	354	91
	18	Lauric acid	2.5	319	15
	21, 22	Lauric acid	2.5	—	—
	23	Lauric acid	2.5	322	25
	28	Lauric acid	2.5	—	—
	29	Lauric acid	2.5	462	42
72	0	Behenic acid	0.5	386	100
	10	Lauric acid	2.5	449	51
	13, 14	Lauric acid	2.5	—	—
	15	Lauric acid	2.5	348	29
	20	Lauric acid	2.5	—	—
	21	Lauric acid	2.5	362	38
74	0	Lauric acid	0.3	714	76
	7	Lauric acid	2.5	682	29
	10, 11	Lauric acid	2.5	—	—
	12	Lauric acid	2.5	525	49
	17	Lauric acid	2.5	—	—
	18	Lauric acid	2.5	681	65*

* Incomplete injection.

0.5% lauric acid solution and 7 parts of the serum and having a pH of 7.9–8.0 was injected intravenously at the dose corresponding to 3 mg/kg of lauric acid. The effects on thrombocytes are demonstrated in Table 5. It is seen that reaction with serum before injection essentially eliminated the effects of the fatty acid on the platelet count. Rabbit serum alone had no effect on the thrombocytes. These results were confirmed in several additional experiments in which the ratio between lauric acid and serum was varied from 1:9 to 4:6. Intraperitoneal injection of 10 mg/kg of lauric acid did not influence the thrombocyte count of rabbits over a 2 hr period.

DISCUSSION

The observation of a rapidly developing but transient thrombopenia after intravenous administration of certain fatty acids leads to several questions. The most important ones refer to the mechanism of action and to the practical significance of increased serum free fatty acids for thrombogenesis in man. One might assume that the intravenously administered fatty acids cause clumping of the thrombocytes and that the platelet aggregates would be retained in the capillary bed, particularly of the lungs. Recent experiments by Soloff and Wiedeman (14), in which thrombus formation could be seen after intra-arterial and intravenous injection of saturated fatty acids in bats, support this assumption. Since the thrombocyte counts had usually returned to the initial values after 1–2 hr, it could be concluded that the fatty acid-induced clumping was largely reversible. However, this assumption is not proven, and it may well be that part of the platelets were destroyed and were rapidly replaced from the reservoirs. The 5-hydroxyindole acetic acid (5-HIAA) levels were therefore determined in the urine of rabbits before and after intravenous injection of 5 mg/kg of myristic acid or lauric acid. Although these

TABLE 3 EFFECT OF FATTY ACIDS OF HIGH PURITY ON PLATELET COUNTS IN RABBITS

Fatty Acid		Purity	Concentration	pH	Dose	Thrombocyte Counts × 10^{-3} Initially	Minutes after Injection 5	10	60	120
			mg/ml		*mg/kg i.v.*	*per mm³*		*% of initial count*		
Stearic acid (Sample II)	(18:0)	>99%*	5	9.0	5	258	44	103	101	—
					5	386	46	60	85	—
					2.5	699	82	97	97	—
					2.5	474	85	96	97	—
Palmitic acid (Sample II)	(16:0)	>99%*	5	9.0	5	449	36	47	72	88
					5	382	24	32	82	91
					2.5	636	73	81	93	—
					2.5	488	25	35	69	71
Lauric acid (Sample II)	(12:0)	>99%*	5	9.5	5	440	27	50	94	—
					5	459	14	27	58	71
					2.5	403	40	72	84	95
					2.5	369	70	89	95	—
Oleic acid (Sample II)	(18:1)	>99%*	5	8.8	10	395	27	57	82	101
					10	384	51	59	100	—
					5	455	67	72	96	—
					5	333	69	73	90	—

* Hormel Foundation, Austin, Minn.

TABLE 4 5-Hydroxyindole Acetic Acid Excretion in the Urine of Rabbits Injected Intravenously with Lauric and Myristic Acids

Rabbit No.	Acid (5 mg/kg)	5-Hydroxyindole Acetic Acid in Urine		
		Bladder Urine before Injection	2 hr-Urine before Injection	2 hr-Urine after Injection
			μg/ml	
62	Lauric	2.7	—	2.6
73	Lauric	4.3	—	0.9
70	Lauric	2.2	1.6	0.4
78	Lauric	10.7	13.9	6.7
69	Myristic	0.8	—	2.3
68	Myristic	10.8	—	1.4
67	Myristic	8.0	9.1	6.0
71	Myristic	4.1	25.0	12.0

TABLE 5 Effect of Added Serum on Thrombocyte Changes in Rabbits after Intravenous Injection of Lauric Acid

Injected Material	Thrombocyte Counts				
	$\times 10^{-3}$ Initially	Minutes after Injection			
		5	10	60	120
	per mm³	*% of initial count*			
Lauric acid (3 mg/kg) as 0.5% injectable solution	356	16	25	68	94
	495	18	43	74	78
Lauric acid (3 mg/kg) mixed with serum	379	93	93	95	—
	494	89	95	102	—
	409	95	97	91	88
Serum (2 ml/kg)	339	95	96	107	—
	344	100	99	98	—

two fatty acids very markedly depress the thrombocyte count at 5 mg/kg, no increase of the 5-HIAA excretion was found. Furthermore, no decrease of the initial platelet counts was seen in three rabbits that had received a total of eight intraveous injections of 0.5 mg/kg of lauric acid each during a 12 day period. A massive destruction of thrombocytes is thus not probable.

The question whether or not the fatty acid-induced thrombus formation in vitro is due to a direct effect of fatty acids on the platelet surface or to activation of the Hageman factor is discussed in detail by Connor (9). The evidence presented favors the assumption that the fatty acid effect is mediated through activation of the Hageman factor. Connor et al. (15) have recently also shown that infusion of fatty acids into anesthetized dogs caused massive thrombosis and rapid death. The authors concluded that intravascular clotting was probably also due to rapid activation of the Hageman factor. Although in our experiments no grossly visible thrombosis was produced, it is conceivable that the transient thrombopenia observed in our rabbits is related to the same mechanism. However, some differences between the results of Connor et al. (15) and our observations are noteworthy, as follows. Extensive thrombosis in dogs was observed only if the fatty acid solutions were turbid. Most of our solutions were clear or became clear when heated before injection. It is thus possible that micelle formation may be responsible for the thrombotic effect described by Connor et al. (15), whereas no micelles were formed after injection of our clear alkaline solutions. Furthermore, no thrombosis was induced in dogs by saturated fatty acids with a chain length of 12 or less (15), whereas in our rabbits, lauric acid was very active. We also observed appreciable activity with unsaturated fatty acids, whereas in the experiments of Connor et al. (15) in dogs and Soloff and Wiedeman (14) in bats, the unsaturated fatty acids caused no thrombosis. In vitro experiments of Margolis (5) showed that long-chain saturated fatty acids are the most potent activators of the Hageman factor, whereas myristic and lauric acids, as well as oleic acid, have slight to negligible effects. This is not consistent with our in vivo observations, and a direct influence of the fatty acids on the platelets is therefore still to be considered. It is also possible that thrombopenia occurred as a consequence of a hemolytic effect of fatty acids, since Hellem (16) has demonstrated that intravenous injection of factor R, which is obtained from hemolyzed erythrocytes and is identical with ADP (17), causes a rapid and transient drop in platelet count in rabbits. Slight hemoglobinuria was observed in some of our animals, but this also occurred after injection of short-chain fatty acids, which had no significant effect on the platelet counts.

The final question, which refers to the significance of increased levels of free fatty acids in the serum as a possible cause for thrombosis, has important implications. There is evidence that occlusive vascular disease may often occur during a period of weight loss when extensive fat mobilization takes place (18). It is also known that under stress conditions, and after administration of epinephrine, serum fatty acid levels are raised (19, 20). Various in vitro and in vivo experiments have shown that the addition of certain fatty acids to the blood may cause thrombocyte agglutination, hypercoagulability or extensive thrombosis. Although the significance of these observations is limited by the low solubility of fatty acids at the physiological pH, these model experiments suggest that increased free fatty acid levels in the blood may be an important pathogenic factor in thrombosis. This assumption is supported by recent findings of Hoak et al. (21) that ACTH and porcine anterior pituitary extract induced a 5- to 7-fold rise in plasma free fatty acids in rabbits that was accompanied by generalized thrombosis. Preliminary experiments in our laboratories have shown that pretreatment with heparin inhibits or reduces fatty acid-induced thrombopenia

in rabbits. This may represent a lead for further research on the mechanism and prevention of thrombosis.

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