THE EFFECT OF CERTAIN FATTY ACIDS ON THE COAGULATION OF PLASMA *IN VITRO.*

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FULLERTON, DAVIE AND ANASTASOPOULOS (1953) showed that following a meal consisting of bacon and eggs, bread and butter, there occurs a significant shortening of the whole blood clotting time in siliconed tubes and of the accelerated plasma clotting time using Russell's viper venom. These results have been confirmed and extended by O'Brien (1955) who showed that the calcium time of plasma is similarly shortened following a fatty meal. Macfarlane, Trevan and Attwood (1941) found that plasma freed from particulate fat by high speed centrifuging showed a considerable prolongation of its clotting time in the presence of Russell's viper venom : the defect could be corrected by replacing the fatty supernatant material removed in the centrifuging process. Thus it seemed reasonable to postulate that the acceleration of coagulation which follows fat feeding might be due to increased numbers of circulating chylomicra. However, in a previous paper (Poole, 1955) it was shown that while it was possible to shorten the calcium time of plasma by adding a washed chylomicron suspension, it was necessary, in order to produce changes comparable with those following fat feeding, to add chylomicra in a concentration greatly in excess of anything which is likely to occur in physiological lipæmia.

The object of this paper is to examine the possibility that free fatty acids contribute to the changes in blood coagulability following fat feeding, by studying the effects of the addition of fatty acids to plasma *in vitro*.

METHODS.

Plasma.—Fresh human plasma has been used. Blood was collected into 3·8 per cent trisodium citrate solution in the proportion of 9 parts of blood to 1 part of citrate solution and the plasma was separated by centrifuging at 4000 rev./min. for 15 min.

Fatty acids.—Experiments have been carried out with the sodium salts of fatty acids. These have been used either as fine aqueous suspensions (prepared by heating the substance with water until it dissolves and then allowing the solution to cool) or as solutions in 5 per cent albumin. Such albumin solutions possibly represent the form in which free fatty acids appear in the circulation under physiological conditions.

Calcium time.—To 0·1 ml. of citrated plasma was added 0·1 ml. of the solution whose activity was being tested in a 63 × 9 mm. test tube standing in a 37° water bath ; 0·1 ml. of 0·025 M calcium chloride solution was then added and the time taken for the mixture to clot was recorded.

Russell's viper venom accelerated clotting time.—To 0·1 ml. of citrated plasma were added 0·1 ml. of the solution whose activity was being tested and 0·1 ml. of 1/10,000 Russell's viper venom (" Stypven ", Burroughs Wellcome) in a 63 × 9 mm. test tube in a 37° water bath ; 0·1 ml. of 0·025 M calcium chloride solution was then added and the time taken for the mixture to clot was recorded.

Thrombin generation test.—The technique of Pitney and Dacie (1953) has been followed.

RESULTS.

A Time-consuming Reaction between Fatty Acids and Plasma.

When a concentration of 3·3 m-equiv./l. of the sodium salt of certain fatty acids is added to plasma, a shortening of the calcium time occurs, to an extent which depends on the time which has elapsed between mixing the plasma with the fatty acid sodium salt and adding the calcium chloride solution. Qualitatively and quantitatively similar results were obtained with aqueous suspensions and

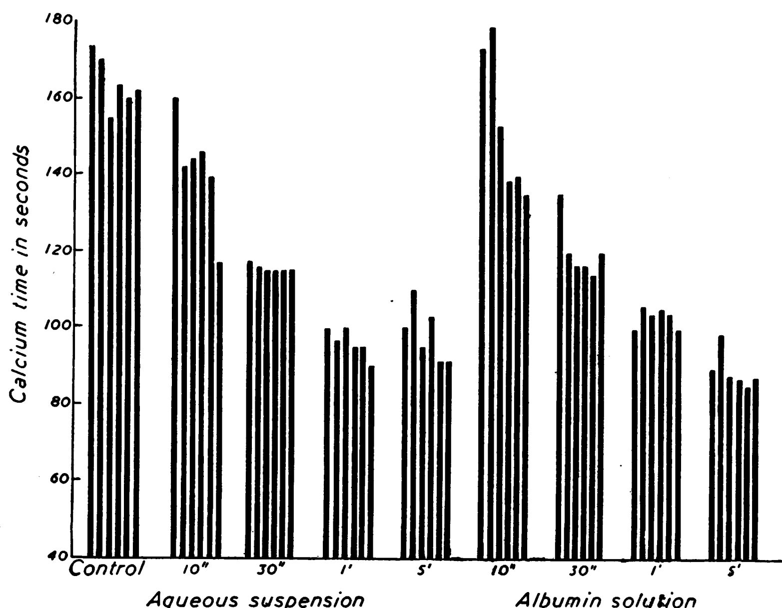

FIG. 1.—Effects of sodium stearate 3·3 m-equiv./l. on the calcium time of normal plasma. To 0·1 ml. citrated plasma was added 0·1 ml. of an aqueous suspension or an albumin solution of sodium stearate. The mixture was recalcified with 0·1 ml. 0·025 M–$CaCl_2$ after pre-incubation at 37° for varying periods of time. The columns represent individual calcium time determinations. Six replicate determinations were made for each set of conditions.

with albumin solutions. Fig. 1 shows the results obtained with sodium stearate. A maximum effect is reached in about 1 min.

Qualitative Differences between Different Fatty Acid Sodium Salts.

Fig. 2 shows the effects obtained with aqueous suspensions of a selection of sodium salts of long chain fatty acids at a concentration of 3·3 m-equiv./l. In each case the suspension was mixed with the plasma 1 min. before recalcification. Stearate, palmitate and oleate produced a substantial acceleration of coagulation, whereas elaidate, linoleate and ricinoleate had no measurable effect. The substances used were described as pure by the manufacturers, but in view of the very considerable difficulties which are encountered in purifying fatty acids, it is not possible to exclude the presence of small amounts of other fatty acids in these

preparations. The following acids have also been tested in a similar manner and have shown no effect on the calcium time: acetic, butyric, valeric, caproic, pelargonic, caprylic, lauric, myristic.

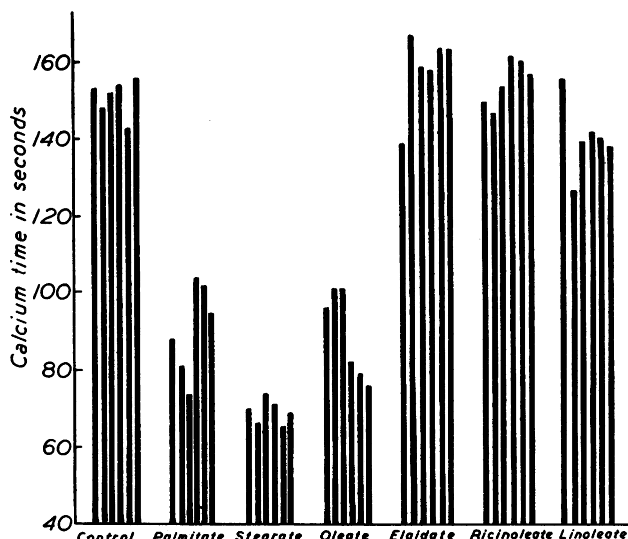

FIG. 2.—Effects of aqueous suspensions of various fatty acid sodium salts (3·3 m-equiv./l.) on the calcium time of normal plasma. A mixture of 0·1 ml. citrated plasma with 0·1 ml. of an aqueous suspension of the fatty acid sodium salt was incubated at 37° for 1 min. and then recalcified with 0·1 ml. 0·025 M–$CaCl_2$. The columns represent individual calcium time determinations.

The Concentration in which Certain Fatty Acid Sodium Salts Accelerate Plasma Coagulation.

A maximal effect is obtained with a concentration of about 3 m-equiv./l. Substantially higher concentrations have an anticoagulant effect. The titration of the effect of sodium stearate given in Table I shows that even with a concentration of 20 μ-equiv./l. a significant effect can be demonstrated.

pH Changes produced by the Addition of Fatty Acids to Plasma.

Stearic acid or sodium stearate added to plasma in a concentration of 3·3 m-equiv./l. produce no change in pH which can be detected with a pH meter. The effect on coagulation is not measurably different whether the test is carried out with the fatty acid, its sodium salt, or the sodium salt to which so much hydrochloric acid has been added as may be necessary to produce neutral pH. It seems, therefore, that pH changes cannot be responsible for the effects described.

Effects of Sodium Salts of Fatty Acids on the Russell's Viper Venom Accelerated Clotting Time.

The sodium salts of certain fatty acids in aqueous suspension at a concentration of 3·3 m-equiv./l. shorten the Russell's viper venom accelerated clotting time of plasma but the results obtained are qualitatively different from the results of

TABLE I.—*Titration of Accelerating Effect of Sodium Stearate on the Calcium Time of Normal Plasma.*

Concentration (m-equiv./l.).	Clotting time (sec.).
0	147, 157
0·02	123, 127
0·04	119, 124
0·06	105, 122
0·08	100, 118
0·10	97, 105
0·20	92, 90
0·40	86, 87
0·60	82, 85
0·80	77, 82
1·0	73, 80
2·0	83, 69
3·0	79, 76
4·0	77, 71
5·0	89, 89

Plasma 0·1 ml. + 0·1 ml. stearate suspension incubated at 37° for 60 sec. and then recalcified with 0·1 ml. 0·025 M–$CaCl_2$. Two replicate determinations at each concentration.

calcium time determinations. With sodium stearate, a time-consuming reaction again occurs : no acceleration is observed when plasma, stearate, venom and calcium chloride solution are mixed as rapidly as possible ; if, however, the plasma-stearate-venom mixture is incubated for a short time before recalcification the clotting time is shortened. However, in addition to this delayed acceleration, an immediate acceleration is obtained with sodium laurate and to a lesser extent with sodium oleate and linoleate. Experimental results illustrating these changes are shown in Table II.

TABLE II.—*Immediate Effects of Sodium Salts of Fatty Acids on Clotting Times Accelerated by Russell's Viper Venom.*

Fatty acid.	Clotting time (sec.).							
Control	16	17	18	17	19	18	17	18
Lauric	10	12	12	11	9	11	10	12
Myristic	17	18	17	18	17	17	17	17
Palmitic	16	17	18	16	16	16	16	16
Stearic	19	18	17	18	17	19	16	18
Oleic	15	14	11	14	14	14	14	14
Elaidic	17	17	17	19	18	19	17	17
Linoleic	14	14	13	15	15	16	14	14

Plasma 0·1 ml. + 0·1 ml. 1/10,000 Russell's viper venom + 0·1 ml. Na-fatty acid suspension (final concn. 3·3 m-equiv./l.) mixed together and recalcified immediately with 0·025 M–$CaCl_2$. Eight replicate determinations for each substance tested.

Effects of Fatty Acid Sodium Salts on Thrombin Generation.

O'Brien (1955) has shown that in human subjects after fat feeding the Russell's viper venom accelerated clotting times are closely correlated with the minimum fibrinogen times obtained in the thrombin generation test, using a technique resembling the modification by Pitney and Dacie (1953) of the procedure introduced by Macfarlane and Biggs (1953). In view of O'Brien's results it was of interest to see whether the effect of sodium laurate on the Russell's viper venom accelerated

clotting time of plasma is paralleled by a shortening in the fibrinogen times recorded in the thrombin generation test. The experiments given in Fig. 3 and 4 show that this is the case : sodium stearate increases the amount of thrombin produced and shortens the time required to reach a maximum concentration of thrombin ; sodium laurate on the other hand, increases the peak concentration of

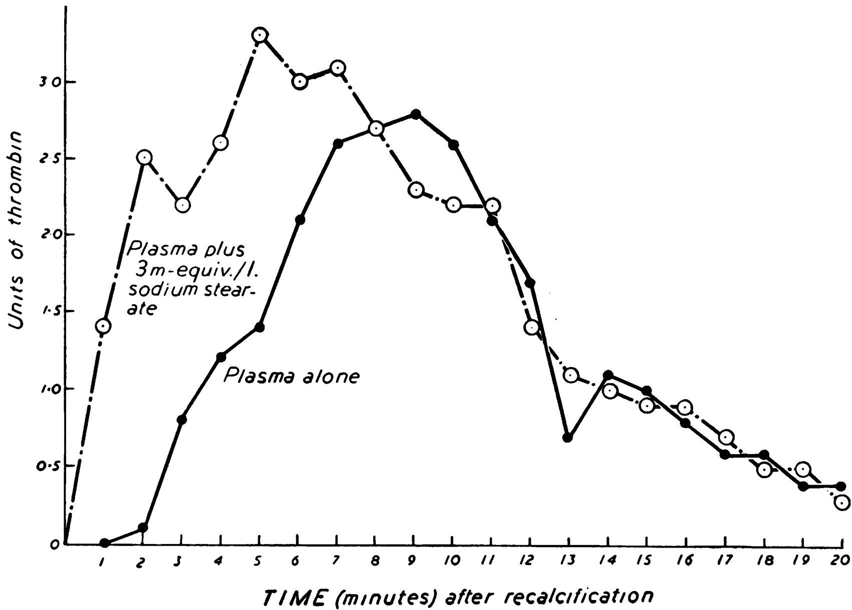

FIG. 3.

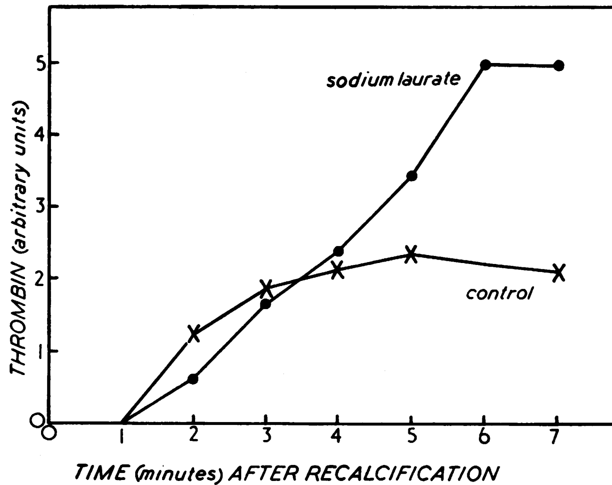

FIG. 4.

FIG. 3 and 4.—Thrombin generation from mixtures of equal vol. of citrated plasma, 3·3 m-equiv./l. fatty acid sodium salt suspension and 0·025 M–$CaCl_2$. The plasma and fatty acid sodium salt suspension were incubated together at 37° for 1 min. before recalcification.

thrombin but does not shorten the time required to reach a maximum concentration.

DISCUSSION.

French, Robinson and Florey (1953) showed that plasma from a rat which had received an injection of heparin would clear added chyle. Robinson and French (1953) found that the clearing phenomenon *in vitro* was accompanied by the conversion of triglycerides into free fatty acid attached to the plasma albumin. Jeffries (1954) demonstrated a similar but weaker clearing activity in the plasma of rats after olive oil feeding, the effect being considerably enhanced by ligating the animal's thoracic duct shortly before exsanguination. Robinson, Jeffries and French (1954) found that the clearing activity produced by fat feeding closely resembles that which follows heparin injection. Hence it is probable that the ingestion of fat is followed by the appearance of free fatty acids in the blood; this supposition receives confirmation from the work of Grossman, Palm, Becker and Moeller (1954) who found that the plasma of fat-fed rats had approximately twice the free fatty acid concentration found in the plasma of starved rats: the increase in concentration was of the order of 0·3 m-equiv./l. Direct evidence of clearing activity in man following fat feeding is so far lacking, but Jeffries (unpublished observations) has demonstrated such activity in a number of other mammalian species and it seems likely that a similar chemical reaction, leading to the production of circulating free fatty acid, occurs in man. If the appearance of free fatty acid *in vivo* has the same effect on coagulation as can be demonstrated by the addition of fatty acids to plasma *in vitro*, then the increase in concentration reported by Grossman *et al.* (1954) would be more than sufficient to account for the increased coagulability of blood produced by fat feeding.

SUMMARY.

Certain fatty acids, in low concentration, appear to accelerate the coagulation time of recalcified citrated human plasma.

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