An Improved Substrate Preparation for Postheparin Plasma Lipase. (31203)

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In recent years, Ediol (Schenlabs Pharmaceuticals, Inc., New York), a coconut oil emulsion, and chylomicra have been generally accepted as substrates for the clearing factor lipase in postheparin plasma(1,2). Other artificial triglyceride emulsions, such as Lipomul (a cottonseed oil emulsion, from Upjohn Co., Kalamazoo, Mich.)(3,4), and olive oil(5,6) have been used but were not as suitable as Ediol. Although chylomicra can be conveniently obtained in chyle collected from cannulated ducts of animals fed a fatty meal (7,8), further processing of the chyle to obtain chylomicra of known triglyceride concentration requires time and effort, and the chylomicra appear to remain stable for only a few weeks even if refrigerated in the presence of small amounts of penicillin and streptomycin(9).

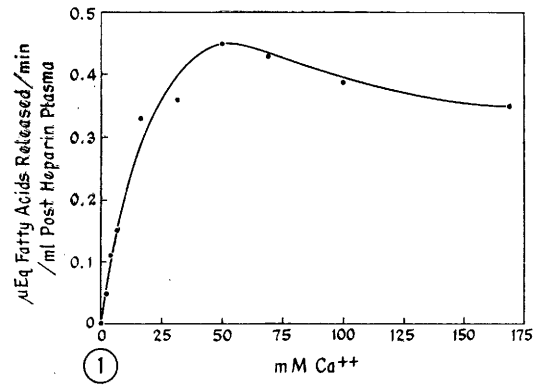
Due to the deterioration of Ediol with time and to the present unavailability of freshly prepared Ediol, it became necessary for us to prepare a substrate emulsion that could be easily assembled from readily available materials. It was reported recently that triolein and tripalmitin were poor substrates for postheparin plasma lipase(10). In this paper, however, we describe a procedure in which purified olive oil (mostly triolein) can be used successfully as a substrate for postheparin lipase, giving approximately 1.3- to 2-fold greater activity than methods previously reported(11).

Materials and methods. Source of plasma. Blood was obtained from human donors just before and 15-20 minutes after intravenous injection of 50 mg Na heparin (Upjohn). Plasmas collected using 0.1 mg heparin per ml as anticoagulant were pooled and kept frozen until used.

Source of substrate. Olive oil (Fisher, USP) was purified *via* alumina column chromatography as previously reported(12). Coconut oil (commercial grade) was obtained from G. T. Walker & Co.; tristearin and tripalmitin (CP) from Fisher; trilaurin from Eastman; trimyristin (99+%), trilinolein (99+%), tripalmitolein (90+%), tricaprin (99+%), tricaprylin (99+%) and tributyrin from Sigma Chemical Co.; and triolein (99+%) from Mann Research.

Preparation of substrate. Kinetic studies described below established the following optimal conditions for preparation of the substrate. 100 mg of purified olive oil (or other lipid substrate) was emulsified in the presence of 6 ml of 0.083 M Tris buffer, pH 8.5, containing 3.3% bovine serum albumin (Armour, Fraction V) and 0.083 M $CaCl_2$, 0.5 ml of 1% Tween 80 (Atlas), 2.0 ml of 1% glycerol and 0.4 ml of water for a total volume of 9.0 ml, the emulsification taking place over 5 minutes while the container rested in a water bath at 40-50°C. The rate of homogenization was controlled through a Variac attachment with the speed set at 60. The Tris buffer mixture was conveniently prepared by mixing equal volumes of 0.25 M Tris, 10% albumin solution, and 0.25 M $CaCl_2$ and adjusting the pH to 8.5. Larger amounts of the above substrate emulsion can also be prepared at one time using multiple ratios of the above formula. When solid triglycerides are used, they must first be liquefied to achieve a satisfactory emulsion. Addition or deletion of any of the above reagents was carried out prior to homogenization of the mixture.

Incubation mixture. The test reaction mixture consisted of 4.5 ml of the above emulsified substrate and 0.5 ml of postheparin plasma. Unless otherwise stated, the final concentrations of the substrate constituents were: 10 mg triglyceride per ml, 50 mM $CaCl_2$, 2% albumin, 0.05% Tween 80 and 0.1% glycerol in 50 mM Tris buffer, pH 8.5. Lipolytic activity was measured by the release of free fatty acids into the incubation mixture as



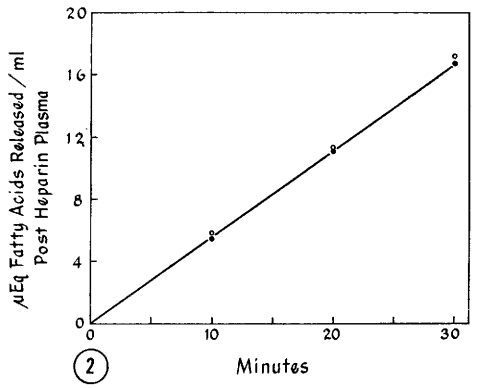


FIG. 1. Effect of Ca^{++} on postheparin plasma lipolytic activity. Substrate concentration was 5 mg of purified olive oil/ml. Other conditions as described in text.

FIG. 2. Rate of hydrolysis of purified olive oil by postheparin plasma. Conditions as described in text. The two sets of points indicate 2 separate determinations carried out one month apart on the same postheparin plasma pool.

follows: Immediately upon adding postheparin plasma to the substrate, 1 ml aliquots were taken and again at 10-minute intervals for 30 minutes and added to 5 ml of the Dole extraction mixture. Fatty acids were estimated by the Dole method(13). Results were expressed as microequivalents free fatty acids released per ml plasma per minute.

Results. Preliminary studies established that there was a wide optimal pH range from 8.2 to 8.6. Although Tween 80 was not essential for activity, Tween 80 eliminated the slight initial lag in hydrolysis that was observed in its absence. Concentrations of Tween 80 above 0.1% were inhibitory. The rate of hydrolysis was not significantly affected by the substrate concentration of olive oil when it varied from 2.5 mg to 20 mg/ml. The presence of albumin above 1% concentration prevented the decreasing rate of hydrolysis with time observed at lower albumin concentrations. At 2% albumin concentration, lipolytic activity was approximately double that in the absence of albumin. Ca^{++} was shown to be obligatory (Fig. 1); however, other ions can be substituted as seen in Table I.

TABLE I. Effect of Various Ions on Lipolytic Activity in Postheparin Plasma.

Ions	Concentration, mM	Activity, μEq FA/min/ml
None	0	0
$CaCl_2$	50	.59
$(NH_4)_2SO_4$	50	.46
"	100	.48
NaCl	50	.50
"	100	.43
$MgCl_2$	50	.55

The linear response to purified olive oil by postheparin lipase indicates that the initial velocity can be readily measured permitting zero order kinetics (Fig. 2). Similar linear responses were also observed with other triglycerides tested. The method was highly reproducible. A hydrolysis rate of 0.54-0.58 μEq fatty acids released/min/ml was observed under optimal or near optimal conditions by a single postheparin plasma pool on at least 6 separate occasions spread over several months. Preheparin plasma gave no lipolytic response in this system.

Table II indicates that the rate of hydrolysis did not appear to depend on degree of saturation or length of the fatty acid chains in the triglyceride molecule. Tristearin and trimyristin could not be properly emulsified under the conditions described above, large but variable amounts of the triglycerides precipitating out upon homogenization. This could account for the lower postheparin lipase activity with these two compounds.

Discussion. Our inability to obtain Ediol readily as a substrate for postheparin lipase activity, and having it, to get reproducible results consistently, prompted us to devise the simple reproducible substrate system described. Our failure to obtain reproducible results with Ediol may have been due to

TABLE II. Rate of Hydrolysis of Various Triglycerides by Postheparin Plasma.

Triglyceride	Activity, μEq FA/min/ml
Purified olive oil*	.56
Coconut oil†	.63
Tristearin	.19
Triolein	.58
Trilinolein	.56
Tripalmitin	.55
Tripalmitolein	.58
Trimyristin	.41
Trilaurin	.54
Tricaprin	.59
Tricaprylin	.55
Tributyrin	.46

* Contains approximately 85% unsaturated fatty acid chain residues(22).

† Contains approximately 84% trisaturated fatty acid chain residues(23).

the deterioration of the emulsion with time or to insufficient "pre-activation" with serum as the preparation aged. Since the nature of this activation process is unknown, the extent of activation of Ediol by serum cannot always be regulated(14).

The work of Korn(15,16) has indicated that the "activation" of an artificial triglyceride emulsion is a prerequisite for postheparin lipolytic activity. Simple triglyceride emulsions were poorly if at all hydrolyzed, but could become available as substrates when preincubated with α-lipoprotein(3). Several groups of workers(2,14), however, have questioned the necessity of this preincubation with serum. The "activation" of Ediol by sonication without prior incubation with serum was demonstrated by Datta(11), suggesting that the "triglyceride-lipoprotein" complex proposed by Korn(1) is not a required feature of postheparin lipolytic activity. Moreover, it has been recently claimed that sonicated aqueous triglyceride suspensions could serve as substrates for postheparin plasma lipase(10). Our findings indeed show that serum preactivation is not necessary. Although albumin is present in the substrate mixture during homogenization, we found that postheparin lipolytic activity was not altered when albumin was omitted during homogenization but added to the reaction system just prior to assay. This suggests that the "triglyceride-lipoprotein or -protein" complex is not a necessary intermediate but that albumin is merely a fatty acid acceptor. In contrast, Datta(11) found that albumin did not increase activity in his system.

Payza *et al*(10) reported that tripalmitin and triolein were poor substrates and that unsaturated triglycerides were hydrolyzed at a slower rate than saturated triglycerides. Our results, however, show that both tripalmitin and triolein are excellent substrates and that the postheparin enzyme showed no preference among esters of fatty acids from C_8 to C_{18}, saturated or unsaturated, confirming observations by Korn(17) for long chain fatty acids (C_{14} to C_{18}) on triglycerides of chylomicrons obtained from rats fed olive oil, corn oil or cream.

Desnuelle and Savary(14) have emphasized the importance of the physical nature of the substrate and have suggested that the general characteristics of the emulsions may have a stronger influence on the rate of lipolysis than the chemical nature of the substrate. A striking illustration is that tristearin is a poor substrate for lipase, not because of the chemical nature of the stearyl chains, but because of its high melting point. Although we were able to emulsify tristearin in our present system at high temperatures, we found that a considerable amount of the triglyceride precipitated out of the emulsion state as the reaction temperature of 37°C was approached. A smaller amount of trimyristin and slight amounts of other triglycerides were also observed to precipitate out of several other saturated triglyceride preparations (C_{12} and above). Despite this slight settling out of longer chain triglycerides, the similarity except for tristearin and trimyristin, of rates of hydrolysis of the various triglyceride emulsions by postheparin plasma lipase, suggests that an optimal emulsion state necessary for maximal lipolytic activity(14) has been achieved.

Shore and Shore(18) reported that triolein, tripalmitin or tristearin in unemulsified form were not hydrolyzed by postheparin plasma. In contrast, emulsified triolein (in the form of olive oil) was hydrolyzed to a small extent (5,6). Workers in Desnuelle's laboratory(14, 19,20) have shown that pancreatic lipase preferentially acts on emulsified esters. More-

over, their work suggests that factors other than emulsification of the substrate alone may also be involved(20). We, too, have found that emulsification of purified olive oil did not assure hydrolysis by postheparin plasma. Thus, no lipolytic activity in postheparin plasma was detected in a turbidimetric system designed for pancreatic lipase(12). In addition, much less activity was observed with the standard titrimetric lipase method of Tietz *et al*(21) than with our present system. All 3 systems employed purified olive oil as substrate but differed in the composition of the substrate mixture and in the method of preparation. This indicates that a certain "mode of presentation" of the substrate to the enzyme as suggested by Desnuelle and Savary(14) is required for maximal activity.

Summary. A rapid, simple, reproducible and readily available substrate preparation for estimating postheparin plasma lipase is described. Ca^{++} is obligatory but can be replaced by other cations such as NH_4^+, Na^+ or Mg^{++}. No preference was shown by postheparin plasma lipase for triglycerides with a particular fatty acid chain length or degree of saturation or unsaturation.

1. Korn, E. D., Meth. Biochem. Anal., 1959, v7, 145.

2. Robinson, D. S., French, J. E., Pharmacol. Rev., 1960, v12, 241.

3. Korn, E. D., Quigley, T. W., J. Biol. Chem., 1957, v226, 833.

4. Kern, F., Steinmann, L., Sanders, B. B., J. Lipid Res., 1961, v2, 51.

5. Suehiro, M., Nakanishi, K., J. Biochem., 1960, v47, 777.

6. Slack, J., Nair, S., Traisman, H., Becker, G., Mahler, S., Hsia, D. Y., J. Lab. Clin. Med., 1962, v59, 302.

7. French, J. E., Robinson, D. S., Florey, H. W., Quart. J. Exp. Physiol., 1953, v38, 101.

8. Havel, R. J., Fredrickson, D. S., J. Clin. Invest., 1956, v35, 1025.

9. Robinson, D. S., Adv. Lipid Res., 1963, v1, 133.

10. Payza, A. N., Eiber, H. B., Danishefsky, I., Abstr. 150th Meeting, A. C. S., Atlantic City, Sept. 13-17, 1965, 103C.

11. Datta, D. V., Proc. Soc. Exp. Biol. and Med., 1963, v112, 1006.

12. Vogel, W. C., Zieve, L., Clin. Chem., 1963, v9, 168.

13. Dole, V. P., J. Clin. Invest., 1956, v35, 150.

14. Desnuelle, P., Savary, P., J. Lipid Res., 1963, v4, 369.

15. Korn, E. D., J. Biol. Chem., 1955, v215, 1.

16. ———, ibid., 1955, v215, 15.

17. ———, in The Enzymes of Lipid Metabolism, Desnuelle, P., Ed., Pergamon Press, New York, 1961, p321.

18. Shore, B., Shore, V., Am. J. Physiol., 1961, v201, 915.

19. Sarda, L., Desnuelle, P., Biochim. Biophys. Acta, 1958, v30, 513.

20. Benzonana, G., Entressangles, B., Marchis-Mouren, G., Pasero, L., Sarda, L., Desnuelle, P., in Metabolism and Physiological Significance of Lipids, Dawson, R. M. C., Rhodes, D. N., Eds., John Wiley & Sons, New York, 1964, p141.

21. Tietz, N. W., Borden, T., Stepleton, J. D., Am. J. Clin. Path., 1959, v31, 148.

22. Eckey, E. W., Vegetable Fats and Oils, Reinhold Pub. Corp., New York, 1954, p724.

23. Deuel, H. H., The Lipids I, Interscience Publishers, Inc., New York, 1951, p232.

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X-Linked Recessive Inheritance of a Syndrome of Mental Retardation With Hyperuricemia. (31204)

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In 1959 two separate case reports described a syndrome of choreoathetosis, cerebral palsy, mental retardation, and self-mutilation in young children with elevated serum uric acid levels(1,2). Lesch and Nyhan recently demonstrated a metabolic abnormality in purine biosynthesis in this syndrome and reported the third affected family(3). Subsequently,