Alkaline phosphatase activity can interfere with the enzymatic determination of triglycerides

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Summary Unlike human plasma, rat plasma was found to contain substantial quantities of alkaline phosphatase. The large amount of phosphatase activity in rat plasma interferes with assay methods that employ orthophosphoric acid esters as substrates or reaction intermediates. Although these methods are effective when applied to human plasma samples, they cannot be used with rodent plasma. —**Dombrowski, G. J., Jr., K. R. Swiatek, and K-L. Chao.** Alkaline phosphatase activity can interfere with the enzymatic determination of triglycerides. *J. Lipid Res.* 1985. **26:** 513–514.

Supplementary key words rat • human • plasma • free fatty acids • phosphate esters

A recent paper (1) has described a problem with the enzymatic determination of free fatty acids in rat and mouse blood. The assay method employed was initially developed for the analysis of free fatty acids in human plasma or serum samples (2). Free fatty acids are first acylated by acyl-CoA synthetase (acid: CoA ligase, EC 6.2.1.3).

$$\text{R-COOH} + \text{ATP} + \text{CoA} \xrightarrow{\text{acyl-CoA synthetase}} \text{R-CO-CoA} + \text{AMP} + \text{PPi}$$

$$\text{AMP} + \text{ATP} \xrightarrow{\text{myokinase}} 2\text{ADP}$$

$$2\text{ADP} + 2\text{phosphoenoylpyruvate} \xrightarrow{\text{pyruvate kinase}} 2\text{ATP} + 2\text{pyruvate}$$

$$2\text{pyruvate} + 2\text{NADH} + 2\text{H}^{+} \xrightarrow{\text{lactate dehydrogenase}} 2\text{lactate} + 2\text{NAD}^{+}$$

In the second step, myokinase (ATP: AMP phosphotransferase, EC 2.7.4.3) catalyzes the transfer of a phosphate group from ATP to the AMP generated during the acylation forming two molecules of ADP. Finally, the production of ADP is coupled to the oxidation of NADH through the actions of pyruvate kinase (ATP: pyruvate 2-*O*-phosphotransferase, EC 2.7.1.40) and lactate dehydrogenase (L-lactate: NAD^{+} oxidoreductase, EC 1.1.1.27). The decrease in NADH concentration is proportional to the initial free fatty acid level and is monitored fluorometrically. When the enzymes and cofactors required for this assay are mixed with either rat or mouse serum, NADH is rapidly destroyed (1). Neither the rate of NADH decomposition nor the extent of NADH loss can be correlated with the free fatty acid concentration determined by alternate means (3).

A similar problem was encountered in this laboratory when a widely accepted clinical method for the determination of triglycerides in human plasma (4) was adopted for measurements in rat plasma. Triglycerides are hydrolyzed by lipase (triacylglycerol acylhydrolase, EC 3.1.1.3) in a triethanolamine buffer at pH 7.6. The liberated glycerol is then phosphorylated by glycerol kinase (ATP:glycerol 3-phosphotransferase, EC 2.7.1.30) and ATP. ADP formed in this reaction is again coupled to NADH oxidation by the addition of pyruvate kinase and lactate dehydrogenase as described above.

$$\text{triglyceride} \xrightarrow{\text{lipase}} 3\text{R-COOH} + \text{glycerol}$$

$$\text{glycerol} + \text{ATP} \xrightarrow{\text{glycerol kinase}} \text{glycerol-PO}_4 + \text{ADP}$$

Attempts to measure triglycerides in freshly prepared rat plasma or recently stored samples using this method failed due to the rapidly decreasing NADH concentration in test reaction mixtures. ATP and phosphoenolpyruvate (PEP) were separately tested and found to be free of ADP or pyruvate contamination. All enzymes used in the assay either exceeded or met the criterion for purity set forth by Wahlefeld (4). Furthermore, NADH was stable in triethanolamine-buffered reaction mixtures containing all required substrates and enzymes until a sample aliquot of rat plasma was added.

In a series of tests where various combinations of substrates and enzymes were employed, it was discovered that NADH, when added alone or in combination with ATP, was stable in the presence of rat plasma. This finding discounts the notion that NADH-destroying substances are present in rat plasma (1). However, immediately after the addition of PEP to a reaction mixture containing only rat plasma and NADH, the NADH concentration began to fall. In contrast, NADH was stable under these same conditions when human plasma was substituted for rat plasma. These results suggest that rat plasma contains a factor(s) that can produce pyruvate from PEP and a sufficient quantity of lactate dehydrogenase to catalyze the reduction of this pyruvate to lactate when NADH is present.

Since the 'NADHase' capacity of rat plasma can be destroyed by exposure to acid or organic solvents (1), the

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reaction appears to be enzyme-catalyzed. In particular, three enzymes could account for the observed decomposition of PEP: an unknown type of nonspecific esterase, pyruvate kinase, and alkaline phosphatase (orthophosphoric-monoester phosphohydrolase, EC 3.1.3.1). Conventional esterase activity (carboxylic-ester hydrolase, EC 3.1.1.1) can be dismissed because phosphoenoylpyruvate is not a carboxylic acid ester. Similarly, endogenous pyruvate kinase cannot be the cause of the problem since only negligible levels of this enzyme were detected in either human or rat plasma (results not shown). Of these possibilities, alkaline phosphatase activity is the most likely cause for the instability of PEP in the presence of rat plasma.

Assay results for alkaline phosphatase in human and rat plasmas are listed in **Table 1**. Phosphatase activity in human plasma was not detectable under these assay conditions, whereas a high level of activity was found in rat plasma. In order to test for the presence of nonspecific esterase activity, ethyl acetate was added to the reaction mixture and its effect on the rate of *o*-nitrophenolphosphate (ONPP) hydrolysis was observed. Since the hydrolytic activity of rat plasma toward ONPP was not suppressed by the addition of 10–20 mM ethyl acetate, the possibility that these samples contained a nonspecific esterase capable of cleaving both carboxylic as well as phosphate acid esters can be ruled out. However, the addition of 5 mM, 10 mM, and 20 mM PEP to the reaction mixtures competitively inhibited the formation of *o*-nitrophenol by 40.1%, 65.3% and 79.1%, respectively. Phosphate, known to be a potent inhibitor of phosphatase (5), decreased ONPP hydrolysis as well as PEP hydrolysis (data not shown) by more than 98% at 50 mM.

TABLE 1. Alkaline phosphatase activity in human and rat plasma

Additions	Human Plasma	Rat Plasma
	μmol/min per ml	
None	0.00	0.158
Ethyl Acetate		
10 mM		0.155
20 mM		0.152
PEP		
5 mM		0.095
10 mM		0.055
20 mM		0.033
Phosphate		
50 mM		0.002

Blood was centrifuged at 3000 *g* for 10 min in order to obtain the plasma fraction. Normal human blood collected during the course of routine laboratory tests (2–3 mg EDTA/ml) was refrigerated 18–24 hr before centrifugation. Rodent blood obtained from 30–60-day-old Sprague-Dawley rats was deposited into heparinized tubes and the plasma was separated immediately. All plasma samples were stored at −60°C and assayed within 2 weeks. Alkaline phosphatase activity was determined by measuring the rate of *o*-nitrophenolphosphate hydrolysis at 30°C in 1 M Tris-HCl at pH 8.0 (6). Each reaction mixture also contained 10 mM $MgCl_2$. The extinction coefficient for *o*-nitrophenol under these conditions is 16,200 cm^2/m at 410 nm. Each value represents the mean of at least three samples.

It is interesting that rat plasma should contain so much more phosphatase than human plasma. Since rat blood cells are fragile in comparison to human erythrocytes, lysed erythrocytes could be the source of phosphatase activity. In order to test this hypothesis, several rat blood samples were hemolyzed to varying degrees by gentle agitation. The hemoglobin-tainted plasma was separated and assayed for phosphatase activity. The results (data not shown) indicate that the phosphatase content of rat plasma is not a function of the extent of hemolysis.

The results of these experiments suggest why the free fatty acid and triglyceride assays described above work with human plasma samples, but not with rat plasma. The alkaline phosphatase in rat plasma hydrolyzes the PEP to pyruvate and inorganic phosphate. Pyruvate in the presence of lactate dehydrogenase rapidly oxidizes NADH to NAD^+.

Although it is possible to subject rat plasma to various treatments which inactivate phosphatase (1), these procedures are cumbersome and can undermine the benefits derived from a totally enzymatic assay. Since the reason these assays do not work well with rat plasma is now known, it is probably best to redesign a method so as not to require the presence of orthophosphoric acid ester substrates. For example, free fatty acids can still be analyzed enzymatically by coupling acyl-CoA synthetase with acyl-CoA dehydrogenase. Similarly, this laboratory now employs a two-enzyme method for triglycerides which couples the action of lipase to that of glycerol dehydrogenase (7).

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