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Plasma ADP Levels: Direct Determination with Luciferase Luminescence Using a Biometer

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PLASMA ADP LEVELS: DIRECT DETEMINATION WITH LUCIFERASE LUMINESCENCE USING A BIOMETER

A method is described for the determination of low plasma levels of adenosine-5'-diphosphate (ADP) using a Dupont Biometer to measure luminescence produced by the luciferin-luciferase reaction. Endogenous ATP is removed by incubation with luciferase. The remaining ADP is then quantitated, following its conversion to ATP, after incubation with creatine phosphate and creatine kinase. The mean coefficient of variation for 0.02 and 2 2 μmol/liter ADP standards were 2.1 and 1.8% respectively. The method has been applied to human and rabbit plasma. Human plasma ADP concentrations were found to be 0.13 ± 0.025 (10) μmol/liter and rabbit plasma concentration were 0.07 ± 0.05 (5) μmol/liter. Several other possible applications of the method are discussed.

EVIDENCE OBTAINED IN RECENT YEARS SUPPORTS THE INVOLVEMENT of adenosine 5'diphosphate (ADP) in the mechanism of platelet aggregation. Thrombocytes can directly be aggregated in vitro by ADP[5] and many believe platelet aggregation is mediated through induced platelet ADP release[7, 9,12]. When blood was passed through a glass bead column platelet retention occurred. This was attributed to the induced ADP release from the red cells and a correlation existed between platelet retention and the amount of hemoglobin concentration in the plasma[20]. Plasma ADP levels may possibly increase in stored blood, or kidney dialyzers and lung oxygenators, due to red cell membrane rupture. This could become a dangerous source to embolic problems. Banked blood was shown to contain an average of 100 aggregates per mm[3], ranging from 10 to 200 μ in diameter[24] and extracorporeal circulation was shown to have a high occurrence of post-operative complications involving vital organs[2, 4, 8]. Platelet aggregation resulting in extensive vascular blockage was demonstrated in endotoxin[19]. The accumulation of high plasma ADP levels could have occurred due to the decreased metabolic state in shock [11, 18, 21] and these levels may have been significant in the platelet aggregation and vascular blockage. Because of the importance shown ADP as a platelet aggregator we felt it appropriate to develop a procedure whereby μmolar plasma ADP concentrations could be analyzed routinely.

Current methods for measuring low levels of ADP do so by first measuring ATP then enzymatically converting ADP to ATP and redetermining ATP[10, 14]. The ADP concentrations are then calculated by difference. Since normal human plasma usually has a relatively high ATP content (2μmol/liter)[11] a low plasma ADP content could be difficult to assess. We therefore, wanted a sensitive method which would directly measure plasma ADP. We chose, as have others,[1, 10, 13-15, 23] the firefly luciferase enzyme system for measuring ATP. The luminescence was measured using a Dupont Biometer[3]. Endogenous ATP was first removed from the plasma samples followed by conversion of ADP to ATP with a creatine kinase (EC 2.7.3.2) system.

METHODS

Extraction procedures.

About 3 ml of venous blood is sampled into a tube to a final anti-coagulant concentration of about 10 mmol/liter disodium ethylendiaminete tracetate per liter. An EDTA concentration of 5 mmol/liter has been shown to totally inhibit the breakdown of adenine nucleotides in plasma[10] as well as preventing coagulation. The whole blood was centrifuged at 2000 x g for 2 min. To the plasma layer was added an equal volume of ethanol/water (96/4 by vol) thus denaturing all enzymes present[10]. The solution was then centrifuged at 12000 x g and the resulting supernate was stored in the refrigerator at 4°C for subsequent analysis.

Luminescence Determinations.

Luciferin luciferase luminescence was determined with a DuPont 760 Luminescence Biometer[3] using DuPont Reagent kits. A 10 μl aliquot of sample or standard was injected into 100 μl of luciferase reaction mixture by means of a springloaded syringe (Shandon ReporJector).

The Biometer digital readout was taken after a period of time. The reaction mixture contained the following: $MgSO_4$, 10 mmol/liter: morpholinopropane sulfonic acid buffer, 10 mmol/liter, pH 7.4: crystalline luciferin, 0.71 mmol/liter and 100 U of crystalline firefly luciferase. The reaction mixture was placed in a Biometer cuvette which was placed in front of the photomultiplier tube inside the instrument. The Biometer can be adjusted so that the digital readout corresponds to the concentration of ATP, or one can construct a standard curve. All standard ATP solutions contained an equal volume of ethanol/water. (96/4 by vol). Ethanol does not interfere with the light reaction.

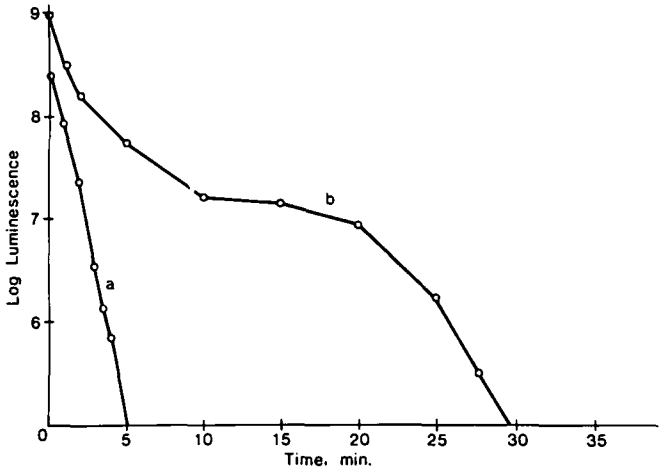

Fig. 1 — Time course of endogenous plasma ATP levels following incubation with luciferin-luciferase. The curves represent 2 μmol/liter and 8 μmol/liter plasma ATP for a and b respectively.

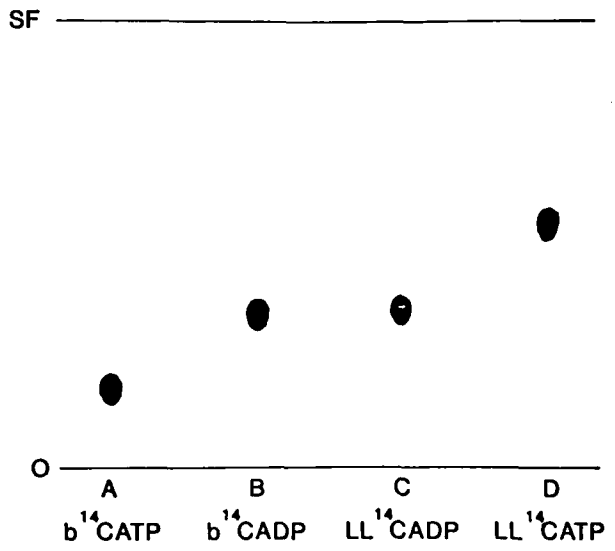

Fig. 2 — Autoradiogram of separated products following incubation of ^{14}C-ATP and ^{14}C-ADP with luciferin-luciferase. A and B are ^{14}C-ATP and ^{14}C-ADP respectively incubated with buffer. C and D are the products after incubation with luciferin-luciferase.

ADP Determinations

In order to utilize the luciferase luminescence for the direct determination of ADP it was first necessary to remove any endogenous ATP.

In a previous[11] paper we accomplished this by incubation of the plasma extracts with apyrase (EC 3.6.1.2), a nuclease from potato. For the present experiments apyrase could not be used because it also hydrolyzes ADP. However, use was made of the specificity of the luciferin luciferase reaction. It has been established[24] that the reaction is specific for ATP and that the product is AMP. Thus, endogenous ATP was eliminated by incubating the plasma extracts with a luciferin-luciferase mixture in an arsenate-magnesium chloride buffer[24] at 25°C. The extent of hydrolysis was followed by reading the luminescence in the Biometer, and the time necessary for the complete removal of ATP was determined.

As reported earlier[11] the highest amount of plasma ATP found was in the rat (8.09 μmol/liter), whereas for rabbits and humans the value is about 2 μmol/liter. Thus 8 μmol/liter ATP was taken as a maximum ATP quantity for removal in experiments involving rats and 2 μmol/liter as the removable quantity when investigating human or rabbit plasma. The actual amount used in the experimental standards was 4 and 1 μmol-liter ATP respectively because of the dilution with ethanol. Into 100 μl of arsenate buffered luciferin-luciferase was injucted 10 of 4 μl and 1 μmol/liter ATP and the luminescence measured. The results, given in Fig. 1, show that a 30 minute incubation period is needed for the complete removal of 4 μmol/liter ATP while the elimination of 1 μmol/liter ATP required only 5 minutes.

We verified the specificity of the reaction using radiolabled ADP and ATP (New England Nuclear, Boston, MA). Ten μl of radioactive ADP and ATP (2000 DPM of each) were mixed separately in 100 μl of arsenate buffered control and 100μl of the arsenate buffered luciferinluciferase mixture. After a reaction time of 20 minutes all four samples were spotted on a polyethyleneimine thin layer plate and separated as described by others[16]. The plates were dried and laid over No-Screen Xray film for 48 hours. After the film was developed the results given in Fig. 2 were obtained. The ATP mixed with the luciferin luciferase (D) showed complete reaction to AMP with no apparent ATP remaining in the vicinity of the standard ATP (A). Also in D there was no detection of any products in the vicinity of the standard ADP position (B). The proximity of the spot in D most likely refers to AMP in accordance with previous results[16]. From the results in C, ADP showed no reaction with the luciferin-luciferase mixture. After the removal of ATP the plasma ADP levels could be assessed using the creatine kinase system:

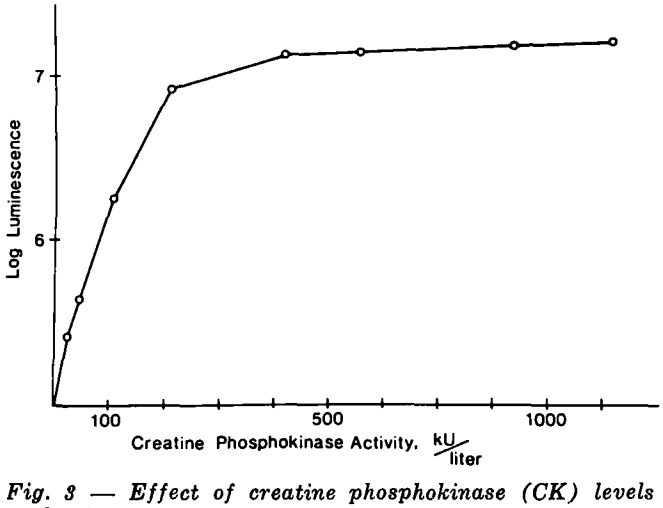

Fig. 3 — Effect of creatine phosphokinase (CK) levels on luminescence.

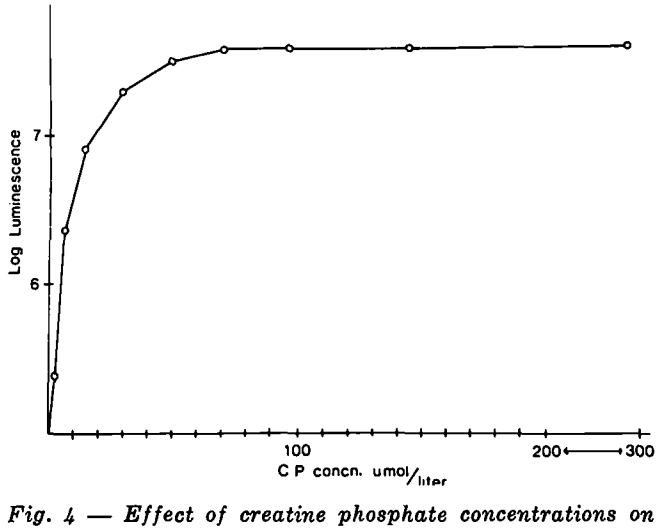

Fig. 4 — Effect of creatine phosphate concentrations on luminescence.

TABLE 1

STABILITY OF ADP IN TWO SAMPLES OF HUMAN PLASMA

Standing Time (Min)	Conc. μ mol/liter A	B
°	2.0	1.96
6	2.0	1.95
11	1.97	1.95
15	1.97	1.93
60	1.95	1.90

Creatine Phosphate + ADP ⇌ Creatine + ATP

The resulting ATP being measured in the Biometer. The amount of creatine kinase (CK) necessary to quantitatively convert the measurable ADP at the expense of a fixed amount of creatine phosphate (CP) was determined. The maximum concentration of CP was arbitrarily chosen as 30 μmol/liter. Since 2 μmol/liter ADP was previously shown to me the critical concentration for platelet aggregation and release[26], this amount was set as the maximum plasma concentration expected. The actual concentration analysed was only 1 μmol/liter since the samples are diluted with 96% ethanol. Strehler's arsenate-magnesium chloride buffer system[24] was used due to CK inhibition by sulfate. The procedure for the determination of the minimum units of CK needed for the assay was as follows; directly into each cuvette containing 100 μl of the luciferin-luciferase mixture was added 10 μl of 30μmol/liter CP and 10 μl of 1 μmol/liter ADP solution in 5 mmol/liter Na_2EDTA and 48% ethanol. Each cuvette was then placed, in turn, in front of the Biometer photomultiplier tube and a 10 μl CK solution injected. The amount of enzyme in the solutions varied from 26 kU/liter to 1680 kU/liter. The digital readout was recorded after 40 seconds at a course sensitivity setting of 6. The results are given in Fig. 3. From this curve the enzyme solution containing 560 kU/liter of CK was chosen.

The concentration of CP necessary for optimum reaction with 1 μmol/liter ADP solution was determined. Varying amounts of CP, from 0.29 to 290 μmol/liter were added to the biometer cuvettes. The procedure was the same as before and 560 kU/liter of CK was injected in each case. Fig. 4 shows that the minimum CP concentration to give a maximum reaction rate was with a 70 μmol/liter solution.

Using the optimal conditions of CK and CP various amounts of ADP were analysed over an 80 second period of time. All ADP standards contained 4 μmolar ATP in 48% ethanol and 5 μmolar EDTA. Ten μl of each standard and 10 μl of 70 μmol/liter CP were injected into the 100 μl of arsenate buffered luciferin-luciferase followed by a 30 minute incubation period at 25°C. Then 10 μl of CK (560 kU/liter) was spring injected into the cuvette and the Biometer reading taken, at a sensitivity of 6, at various times. The results are given in Fig. 5. The readout time chosen for ADP quantitation was 40 seconds, which allows adequate time for the reaction to equilibrate. Fig. 6 is an ADP standard curce, over a concentration span of 100 fold, using the 40 second time.

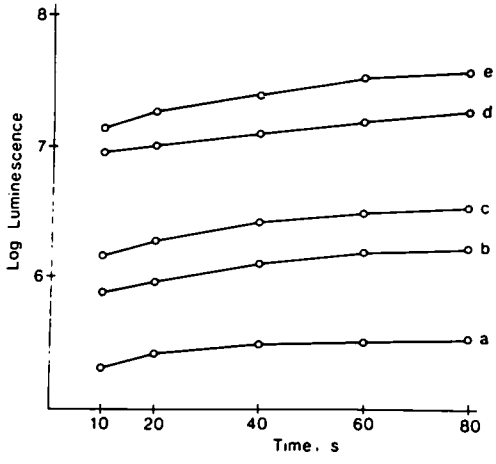

Fig. 5 — Effect of time of reading the Biometer after injecting the sample for determining ADP. ADP concentrations (μmol/liter) were: a, 0.02; b, 0.10; C, 0.21; d, 1.04; c, 2.08.

TABLE 2

HUMAN AND RABBIT PLASMA ADP LEVELS.

ADP Source	μ mol/liter ADP
Normal Human	0.130 ± 0.025 (10)
Normal Rabbit	0.07 ± 0.05 (5)
Rabbit in Endotoxin Shock	0.26 ± 0.13 (5)

ADP Stability.

The stability of ADP in human plasma following the initial centrifugation was assessed. Because of the normally low ADP levels each sample was made to a theoretical ADP concentration of 2 μmol/liter using a standard ADP solution. Two samples of human plasma were allowed to stand, at room temperature, and aliquots were extracted and the ADP levels determined at the various time intervals shown in Table 1.

As can be seen there was essentially complete ADP retention over a 60 minute standing period.

RESULTS AND DISCUSSION

The method as described is such that the measurements can be made of ADP levels over a wide range of concentrations. In this paper we used a concentration span of 100 fold, the linearity of which is seen in Fig. 6. Day to day precision was evaluated using three determinations of each standard, ranging from 0.02 to 2 μmol/liter, every day the Biometer was used. The overall average of the mean coefficient of variations for all assays was 1.75%. The precision of a single sample was assessed by extracting two control sera, with adjusted ADP levels, 20 times and measuring the luminescence in the usual manner. The mean coefficient of variation for 0.02 and 2 μmol/liter ADP were 2.1 and 1.8% respectively.

We have applied the method to human and rabbit plasma. The human samples were obtained from

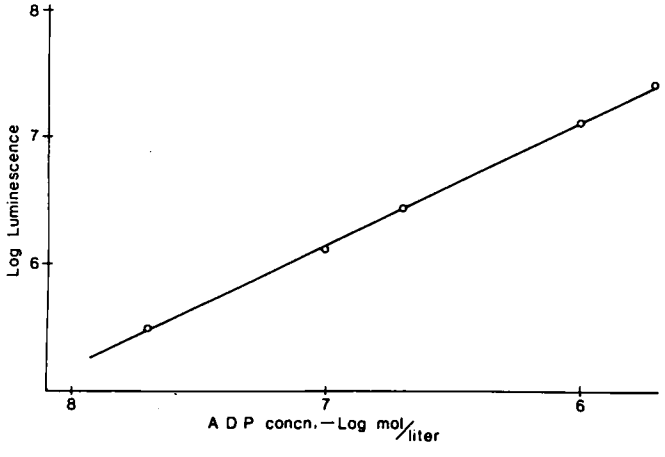

Fig. 6 — Typical ADP standard curve.

laboratory technicians. Rabbit samples were obtained from normal rabbits before and at death due to E. coli enduced endotoxin shock. These results are given in Table 2. The plasma ADP levels of the rabbits in endotoxin shock increased almost 4 fold, however, they never reached the 1 to 2 μmol/liter concentration shown necessary to aggregate platelets *in vitro* using platelet rich plasma[26]. We do not immediately know the explanation for this but the phenomenon is currently under investigation.

Additional applications of the present method are also feasible. In atherosclerosis the vessel walls narrow until total occlusion results and individuals who have this problem usually die of strokes or coronaries, because of eventual thrombotic complications. Elevated ADP plasma levels in these patients could be a causative factor leading to platelet wall deposits instigating the narrowing process. Atherosclerotics are extremely sensitive to ADP as shown by the fact that ADP concentrations which normally cause only a primary aggregation in normal individuals cause a high primary wave or secondary wave in these patients[27]. It could prove beneficial to monitor the plasma ADP levels in these patients. The ADP content of stored blood should possibly be periodically checked before being used in transfusions because of possibly high ADP levels which may develop during storage and be detrimental to the recipients. The increasing use of artificial systems to replace impaired organs, e.g., dialyzers for kidneys or oxygenators for lungs were shown to become impeded by adhering aggregates of platelets[6]. However, the reason for occurrence has not been definitely determined Should hemolysis occur in these procedures, because of the mechanical shear on the red cells, one would have to be suspicious of the ADP level of the perfused blood. In open heart surgery, accompanied by cardiopulmonary bypass, periodic sampling of blood levels of ATP, ADP and CP from the heart pump oxygenator may give an indication of the metabolic cellular condition of the patient.

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