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A Simple Fluorimetric Microassay for Adenine Compounds in Platelets and Plasma and its Application to Studies on the Platelet Release Reaction

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1. The formation of a stable fluorescent product between chloroacetaldehyde and adenine or its derivatives provides the basis of a rapid simple assay for total adenine compounds in blood platelets and in plasma. The assay will measure down to 200pmol of adenine nucleotides. An evaluation of the method established the optimum conditions for the production of maximum fluorescence. 2. Values obtained for total adenine compounds in platelets were 12.9nmol/10^8 cells in man and 7.8nmol/10^8 cells in rat. These closely agree with previous values for total platelet adenine nucleotides found by using a firefly luciferase assay, or a recycled NAD-linked photometric assay. This supports the concept that the chloroacetaldehyde reaction measures total adenine nucleotides in platelets. 3. Platelet aggregation induced by collagen was studied photometrically in 0.1ml volumes of citrated platelet-rich plasma, and total adenine nucleotides were assayed in platelets and plasma before and after aggregation. During aggregation 58% of adenine nucleotides were released from human platelets, and 36% from rat platelets. 4. The chloroacetaldehyde assay is no substitute for more sophisticated procedures, but is a simple sensitive means of monitoring the release of adenine nucleotides from blood platelets and is particularly valuable when small plasma samples must be used.

Platelet aggregation induced by collagen or thrombin is accompanied by a 'release reaction' (Grette, 1962), which involves specific extrusion of adenine nucleotides from subcellular granules (Mills *et al.*, 1968). The most widely-used method of measuring release of platelet adenine nucleotides is the firefly luciferase assay (Holmsen *et al.*, 1966), which is a lengthy procedure, but can measure individual nucleotides after their conversion into ATP. It has been shown that adenine and its derivatives form a stable fluorescent product with chloroacetaldehyde (Kochetkov *et al.*, 1971; Avigad & Damle, 1972). We have developed a rapid simple assay on the basis of this reaction, which measures the total adenine compounds contained in, and released by, blood platelets, in platelet-rich plasma samples of 0.1ml or less. In the present paper we evaluate the method and describe the results of experiments in which adenine release was measured during collagen-induced platelet aggregation in citrated platelet-rich plasma from man and rat.

Methods

Chloroacetaldehyde

Chloroacetaldehyde dimethyl acetal (R. N. Emanuel Ltd., London S.E.1, U.K.) was distilled from 8% (v/v) H_2SO_4, yielding chloroacetaldehyde (b.p. 85–86°C), which could be kept at 4°C for at least 3 months without loss of activity. This was dissolved in distilled water to give a 0.5M solution immediately before use.

Collagen

Collagen suspension was prepared from human subcutaneous connective tissue by the method of Zucker & Borrelli (1962). The hydroxyproline content of this was measured (Bergman & Loxley, 1963) and the value obtained was multiplied by seven to give the 'collagen protein' content of the preparation.

Preparation of platelet-rich plasma

Human blood was obtained by antecubital venepuncture from volunteers who had allegedly taken no drugs during the previous week. Rat blood was obtained from animals anaesthetized with ether, by venepuncture of the inferior vena cava. Samples were anticoagulated with trisodium citrate (final concentration in blood 3.13mg/ml), and centrifuged at room temperature for 10min at 180*g*. The supernatant platelet-rich plasma was removed and platelets in it were counted with a Coulter model B particle counter.

Platelet aggregation

Aggregation was studied photometrically (Born, 1962) in 0.1ml volumes of platelet-rich plasma, by

using a Bryston Aggregometer with modified light-path and sample holder. Samples were incubated for 1min at 37°C, then stirred for 4min at 800 rev./min, with or without 10μl of connective tissue suspension, which contained 43.4μg of collagen protein/ml. Responses were measured as the maximum rate of change of optical transmission. Non-stirred control samples were incubated for 5min at 37°C.

Platelet-free plasma

Blood samples were centrifuged at 10000*g* for 20min and the supernatant plasma was removed. This was essentially free of platelets. The protein content of platelet-free plasma samples was measured by the method of Lowry *et al.* (1951), with bovine serum albumin (Sigma Chemical Co., Kingston-upon-Thames, Surrey, U.K.) as standard.

Assay of adenine and derivatives in platelets and plasma

Platelet-rich plasma samples were diluted with 0.2ml of 0.2% (w/v)EDTA in iso-osmotic saline, and centrifuged for 15min at 3500*g*. The supernatants were removed and the platelet pellets resuspended in 0.25ml of distilled water. Proteins in the resuspended pellets and in the supernatants were precipitated with 50μl of ice-cold 1.0M-trichloroacetic acid and sedimented by centrifugation at 3500*g* for 10min. This procedure extracts virtually all of the adenine nucleotides from platelets (Holmsen, 1972). A sample (0.2ml) was taken from each supernatant and heated for 30min at 100°C with 0.5ml of 0.5M-chloroacetaldehyde and 0.5ml of 0.2M-sodium phosphate buffer, pH6 (Dawson *et al.*, 1969). The tubes were then cooled on ice, and a further 0.5ml of buffer and 2ml of chloroform were added. After vortex mixing and phase separation by centrifugation, fluorescence in 1ml volumes of the aqueous phase was measured by using a Farrand mark 1 spectrofluorimeter. Excitation and emission wavelengths (uncorrected instrument readings) were 310nm and 410nm respectively.

Calibration curve for adenine assay

Solutions of ADP and ATP in distilled water were prepared, such that 5μl added to 0.1ml of platelet-free plasma and assayed by the standard procedure, gave amounts in the fluorimeter cuvette of between 0.20 and 5.0nmol. Plasma blanks (without added nucleotide) were included and photomultiplier readings in the test samples were corrected for readings in the blanks. Blank readings were always less than 0.01μA. Concentrations of nucleotides in the standard solution were checked by absorbance at 258nm ($\epsilon = 15400$ litre$\cdot$mol$^{-1}\cdot$cm^{-1}).

Results and Discussion

Calibration curve

Fluorescence produced by either ADP or ATP with chloroacetaldehyde was identical (Fig. 1). Additional experiments established that the fluorescence/concentration relationship was linear up to 50nmol, which was the highest amount tested. Unless otherwise stated, the values refer to the amounts present in the fluorimeter cuvette at the end of the assay procedure.

pH values of reaction mixture

Kochetkov *et al.* (1971) found that the optimum pH value for the initial rate of formation of the fluorescent product was 4.5; increasing or decreasing this value by 1 pH unit markedly decreased the rate. Results from our laboratory (J. L. Gordon & A. H. Drummond, unpublished work) confirmed this, but when the fluorescence after 30min of incubation at 100°C was measured (instead of the initial-reaction rate) the pH optimum values extended from 3.0 to 4.5, and higher values (up to 5.8) resulted in only a slight decrease in fluorescent yield. At pH values below 3.0, however, the response decreased sharply (Fig. 2).

Incubation time and chloroacetaldehyde concentration

All our experiments were performed at an incubation temperature of 100°C, because the reaction rate is directly related to temperature; for maximum fluorescence with an incubation temperature of 37°C, an incubation time of 24h is needed (Barrio *et al.*, 1972). The time of incubation was varied between

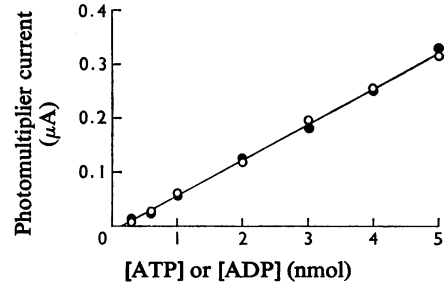

Fig. 1. *Calibration curve for the fluorescent product formed by incubating adenine nucleotides with chloroacetaldehyde*

Either ATP (○) or ADP (●) was added to 0.1ml of platelet-free plasma, which was diluted with 0.2ml of iso-osmotic saline containing 0.2% (w/v) EDTA. Proteins were precipitated with trichloroacetic acid (50μl; 1.0M), and 0.2ml of each supernatant was incubated at 100°C for 30min with chloroacetaldehyde (0.5ml; 0.5M) and phosphate buffer (0.5ml; pH6; 0.2M). Fluorescence was measured in 1ml volumes, which contained 0.2–5.0nmol of the nucleotide–chloroacetaldehyde product.

10 and 60min, but 0.5ml of chloroacetaldehyde solution was always added to each sample. When 0.5M-chloroacetaldehyde was used, fluorescence increased during the first 30min of incubation, and declined after 40–60min (Fig. 3). The decrease in intensity of fluorescence coincided with the appearance of a pale-yellow colour in the reaction mixture, indicating colour-quenching of the fluorescence. When 1.0M-chloroacetaldehyde was used, quenching occurred sooner and was more pronounced. With 0.2M-chloroacetaldehyde, no quenching was seen, but maximum fluorescence was not obtained (Fig. 3). Therefore as a routine we used 0.5M-chloroacetaldehyde and incubated samples for 30min. The colour reaction not only increased with the chloroacetaldehyde concentration, but was greatest when samples containing no adenine were incubated, indicating that free chloroacetaldehyde was responsible. Kochetkov *et al.* (1971) reported that decomposition and condensations of chloroacetaldehyde occurred at neutral or slightly acid pH values, a process that became marked with increasing temperature.

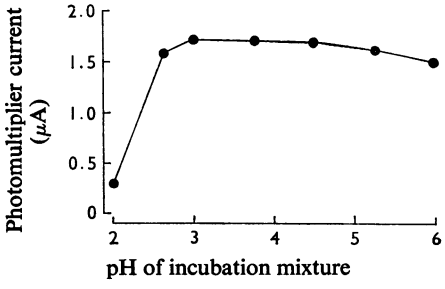

Fig. 2. *Effect of the pH value of the incubation mixture on ADP–chloroacetaldehyde fluorescence*

ADP was added to 0.1ml of platelet-free plasma, which was diluted with 0.2ml of iso-osmotic saline containing 0.2% (w/v) EDTA. Proteins were precipitated with trichloroacetic acid (50μl; 1.0M), and 0.2ml of the supernatant was incubated at 100°C for 30min with chloroacetaldehyde (0.5ml; 0.5M) and phosphate buffer (0.5ml; pH6; 0.2M). The pH value of the solution was adjusted before incubation by the addition of more trichloroacetic acid. Fluorescence was measured in 1ml volumes, each containing 25nmol of the ADP–chloroacetaldehyde product.

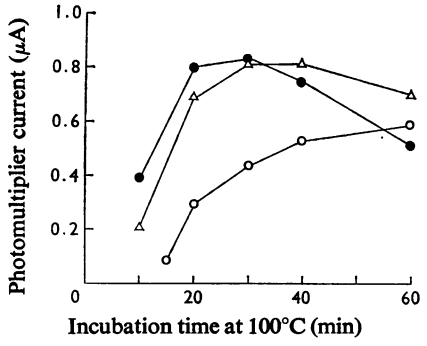

Fig. 3. *Effects of incubation time and chloroacetaldehyde concentration on ADP–chloroacetaldehyde fluorescence*

ADP was added to 0.1ml of platelet-free plasma, which was diluted with 0.2ml of iso-osmotic saline containing 0.2% (w/v) EDTA. Proteins were precipitated with trichloroacetic acid (50μl; 1.0M), and 0.2ml of the supernatant was incubated at 100°C for 10–60min, with chloroacetaldehyde (0.5ml) at 0.2M (○), 0.5M (△), or 1.0M (●) concentrations, and phosphate buffer (0.5ml; pH6; 0.2M). Fluorescence was measured in 1ml volumes, each containing 15nmol of the ADP–chloroacetaldehyde product.

Plasma-protein precipitation and buffering of the supernatant

Preliminary tests established that adequate precipitation of proteins in 0.1ml of plasma (8–9mg of protein) required a final trichloroacetic acid concentration greater than 0.1M. The protein content of a platelet button from 0.1ml of plasma, plus residual plasma around the button, is about 1mg. In our standard assay procedure trichloroacetic acid at a final concentration of 0.17M was used, which ensured satisfactory protein precipitation in all platelet buttons and supernatant plasmas. After precipitation of plasma proteins by trichloroacetic acid, pH values in the supernatant were lower than 3.0 and a series of phosphate buffers was tested to determine the optimum conditions for keeping the pH value of the reaction mixture within the required range (pH3.0–4.5).

Standard samples (0.3ml) were prepared from platelet-free plasma mixed with the EDTA–saline solution; these contained either 9.6 or 0.9mg of protein, i.e., the protein content was just above the plasma values, or just below platelet values respectively. To establish the optimum buffer strength each sample was precipitated with trichloroacetic acid and then 0.5ml of phosphate buffer (0.1, 0.2, 0.3 or 0.5M), pH6, was added to 0.2ml of each supernatant. The pH value of the reaction mixture was measured after addition of 0.5ml of chloroacetaldehyde (0.5M). Control of the pH value was unsatisfactory with 0.1M buffer, but buffers of 0.2–0.5M kept the reaction mixture between pH3 and 6.

ADP was added to a second group of samples containing 9.6mg of protein. The experiment with a range of buffer strengths was repeated, and fluorescence in the supernatants was measured after incubation. In samples containing 0.3M and 0.5M buffer, quenching was seen after 10–20min of incubation, and maximum fluorescence was never

obtained (Fig. 4). In this experiment, the 0.1 M buffer was satisfactory because the high initial protein content decreased the free acid in the supernatant. When the experiment was repeated with low protein (1 mg) samples, fluorescence in samples containing 0.1 M buffer was submaximal because the pH value of the reaction mixture was less than 3. We therefore used 0.2 M buffer as a routine, which ensured that in samples containing 0.9–9.6 mg of protein the pH values of the supernatants remained within the required range, but no colour-quenching occurred after 30 min of incubation. A further 0.5 ml of buffer was added after incubation to ensure that the aqueous phase was always approximately pH 6, the optimum pH value for fluorescence emission (Secrist *et al.*, 1972). Changes in the strength of this diluting buffer did not affect the fluorescence.

Platelet aggregation and release of adenine nucleotides

Stirring alone caused no detectable aggregation or release in either human or rat citrated platelet-rich plasma. The mean aggregation rate after addition of collagen (4.3 μg/ml) was 117 ± 9 S.E.M. in man and 82 ± 5 S.E.M. in rats (10 subjects in each group).

The content of total adenine compounds in platelets was 12.9 nmol/10^8 cells in man and 7.8 nmol/10^8 cells in rat. After collagen-induced aggregation, the content fell by 58% in man, and by 36% in rat, but not all of the released adenine compounds were detectable in the supernatant plasma (Table 1).

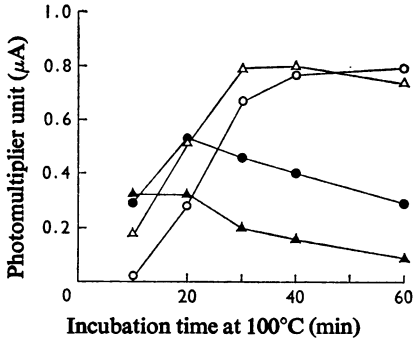

Fig. 4. *Effects of buffer strength and incubation time on ADP–chloroacetaldehyde fluorescence*

ADP was added to 0.1 ml of platelet-free plasma, which was diluted with 0.2 ml of iso-osmotic saline containing 0.2% (w/v) EDTA. Proteins were precipitated with trichloroacetic acid (50 μl; 1.0 M), and 0.2 ml of the supernatant was incubated at 100°C for 10–60 min, with chloroacetaldehyde (0.5 ml; 0.5 M) and phosphate buffer (0.5 ml; pH 6) at 0.1 M (○), 0.2 M (△), 0.3 M (●), or 0.5 M (▲) concentrations. Fluorescence was measured in 1 ml volumes, each containing 15 nmol of the ADP–chloroacetaldehyde product.

During the platelet release reaction, some of the adenine nucleotides in platelets are converted into IMP, inosine and hypoxanthine (Ireland, 1967). These deaminated metabolites do not react with chloroacetaldehyde (Secrist *et al.*, 1972), and it seems probable that the smaller values which we obtained for total adenine nucleotides in platelets plus plasma after aggregation were due to the metabolic deamination which accompanies release.

To determine the reproducibility of the assay method in replicate analyses, the adenine content of platelets from 10 rats was measured. Five or six replicate samples from each animal were tested, and the percentage deviation of each result from the mean value for the animal in question was calculated. The largest individual deviation was 7.5%, and the mean deviation for the entire group was 3.38%.

Comparison with other assay methods

Previous measurements of platelet adenine nucleotides have most commonly been made with a firefly luciferase assay (Holmsen *et al.*, 1966), although a recycled NAD-linked photometric assay (Mills & Thomas, 1969) and simple absorbance at 260 nm (Mürer, 1969) have also been used. Chloroacetaldehyde combines with adenine and its derivatives in a 1:1 stoicheiometric ratio and a fluorophore is produced by alkylation at adenine *N*-1 followed by ring closure

Table 1. *Total adenine compounds in platelets and plasma before and after platelet aggregation by collagen*

Samples (0.1 ml) of citrated platelet-rich plasma were diluted to 0.3 ml with 0.2% (w/v) EDTA in iso-osmotic saline after incubation at 37°C without stirring or after stirring at 37°C with 0.43 μg of collagen. Platelets were sedimented and adenine compounds in platelets and plasma determined. For details see the Methods section. Values given are means ± S.E.M. Four samples of 0.1 ml of platelet-rich plasma from each of 10 subjects were tested. Mean platelet count/ml: Man $3.2 \times 10^8 \pm 0.2$; Rat $14.4 \times 10^8 \pm 0.5$.

	Adenine (nmol/10^8 cells) in platelet-rich plasma		
	Non-stirred In platelets alone	+ Collagen	Decrease (%) after aggregation
Man	12.9 ± 0.49	5.4 ± 0.31	58
Rat	7.8 ± 0.22	5.0 ± 0.19	36
	In supernatant plasma		
Man	2.6 ± 0.25	8.1 ± 0.56	
Rat	0.9 ± 0.04	2.1 ± 0.08	
	In platelets plus plasma		Recovery (%) after aggregation
Man	15.6 ± 0.37	13.5 ± 0.43	86
Rat	8.7 ± 0.13	7.1 ± 0.14	82

and elimination (Secrist *et al.*, 1972), resulting in the formation of an etheno bridge between the *N*-1 and the amino group of the adenine moiety. The amounts of free adenine and adenosine in platelets are negligible (Holmsen & Rozenberg, 1968) and therefore the chloroacetaldehyde assay should measure total adenine nucleotides in platelets, although the reaction is based on the adenine moiety rather than the nucleotides themselves. The values obtained in the present study for the content of 'adenine nucleotides' in human platelets (12.9nmol/10^8 cells) agree with previous estimations made with the luciferase or NAD-linked assays (Mills & Thomas, 1969; Holmsen, 1972), apart from values obtained by extraction of platelet nucleotides with ethanol (Holmsen *et al.*, 1969), which does not extract all platelet adenine nucleotides (Holmsen, 1972). Our value for rat platelets (7.8nmol/10^8 cells) closely agrees with that (8.1nmol/10^8 cells) reported by Mills & Thomas (1969). These results support the concept that the chloroacetaldehyde reaction measures total platelet adenine nucleotides, and indicate that the method is a simple and rapid means of monitoring the platelet release reaction. Its sensitivity makes it valuable when small plasma samples must be used, although it is no substitute for assay methods that measure individual nucleotides.

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