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Adverse Effect of Gel Filtration on the Function of Human Platelets[1,2] (38050)

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Platelets can be separated from nonabsorbed plasma constituents by filtration on Sepharose 2B (1). Platelets isolated in this manner are morphologically and ultrastructurally intact (2), and retain their sensitivity to adenosine diphosphate (ADP) (1–3). These findings prompted Levy-Toledano *et al.* (3) to advocate their use in the clinical study of functional platelet abnormalities. Little is known, however, of gel filtered platelet's (GFP) ability to participate in the release reaction. Since clinical evaluation of platelet dysfunction requires assessment of both the platelet's ability to respond as well as to release ADP, we examined the functional characteristics of GFP after stimulation with weak collagen suspensions and epinephrine.

Materials and Methods. Gel filtration on Sepharose 2B (Pharmacia Fine Chemicals, Uppsala, Sweden) was performed as described by Tangen *et al.* (1) using the following eluting agents: (1) 0.154 *M* NaCl; (2) a noncolloidal buffer composed of 0.141 *M* NaCl, 0.0154 *M* Tris-HCl (pH 7.4), 0.1% D-glucose, 5.35×10^{-2} *M* KCl, 9.8×10^{-4} *M* $MgCl_2 \cdot 6H_2O$ and 6.01×10^{-6} *M* $CaCl_2$; and (3) noncolloidal buffer plus 1.6% albumin. Sepharose 2B, from which the fines had been removed by sedimentation, was made up in suspension with the eluting agent and poured into 1.5 cm × 30 cm siliconized glass columns. Several bed volumes of eluting agent were run through the gel columns before use.

Platelet aggregation was measured turbimetrically in citrated platelet-rich plasma (PRP) in response to ADP (final concentration in PRP, 2×10^{-5} *M*), suspensions of homogenized collagen, and epinephrine (final concentration in PRP, 5.5×10^{-6} *M*) as previously described (4). Before aggregation, GFP and PRP were diluted to 300,000 platelets per mm^3 with platelet-poor plasma (PPP). Percent aggregation refers to the percent change in light transmittance 4 min after the addition of the aggregating agent. All studies were carried out within one hour of blood collection.

In labelling experiments, 2 ml of PRP was incubated with 0.5 μCi of adenine-8-^{14}C (Schwarz/Mann, 56 mCi per mmol) for 90 min, gel filtered, and extracted with 0.8 *M* perchloric acid. The precipitate was packed by centrifugation at 4°C and washed with 0.4 *M* perchloric acid. The combined extract and washing were neutralized with 30% KOH and the precipitated potassium perchlorate removed by centrifugation. Adenine nucleotides present in the extract were separated by chromatography on Dowex 1-X10 (200–400) using a modification of the method of Warner and Hobbs (5). Radioactivity was determined by liquid scintillation counting and corrected for self-absorption. ADP and adenosine triphosphate (ATP) were analyzed in GFP and PRP by a modified firefly luceriferase technique (6).

[1] The opinions or assertions contained herein are the private views of the authors and are not to be construed as official or as reflecting the views of the Department of the Army or the Department of Defense.

[2] Presented in part at the IVth International Congress on Thrombosis and Haemostasis, Vienna, Austria, June 1973.

TABLE I. The Aggregation of Gel-Filtered Platelets % Aggregation.[a]

Agg. Agent	NaCl	Eluting agent buffer	Buffer-Albumin
ADP	66	56	100
Collagen	21	27	31
Epinephrine	11	7	4

[a] % of aggregation observed with PRP.

Results and Discussion. While our studies confirm earlier reports that platelets eluted with buffer containing albumin retain sensitivity to ADP, GFP did not aggregate normally in response to either collagen or epinephrine (Table I). Since collagen-induced aggregation is preceded by and results from adenine nucleotides released from the platelets (secondary aggregation) (7), we studied the release of ADP and ATP from GFP during aggregation with this aggregating agent. When compared with PRP controls, significantly less ADP ($P < 0.01$) and ATP ($P < 0.01$) were released by GFP. No correlation existed between nucleotide release and the eluting agent (Table II). Similar results were observed with epinephrine.

Although this failure to release adenine nucleotides accounts for the unresponsiveness of GFP to collagen and epinephrine, these data do not distinguish between damage to the platelet release mechanism and loss of nucleotides during filtration. Nucleotide content of GFP was therefore compared with PRP controls. As shown in Table III, loss of both ATP and ADP occurred during passage through the gel column with each of the eluting agents.

TABLE II. The Release of Nucleotides by Gel-Filtered Platelets in Response to Collagen.

	ATP[a]	ADP[a]
PRP-control	0.95	1.45
GFP		
Eluting agent		
0.154 *M* NaCl	0.13	0.17
Buffer	0.28	0.37
Buffer–Albumin	0.23	0.29

[a] nmoles/10^8 platelets.

TABLE III. Gel Filtration Effect—% Nucleotide Loss.

Elution	ATP	ADP
0.154 *M* NaCl	31.6 ± 6.0%	46.6 ± 6.8%
Buffer	51.0 ± 14.7%	40.7 ± 2.3%
Buffer–Albumin	59.5 ± 17.0%	36.4 ± 7.8%

Platelet adenine nucleotides are distributed in two pools: (1) a nonmetabolic or storage pool stored in subcellular granules, and (2) a metabolic pool located in the cytoplasm, membranes, and mitochondria (8). During aggregation, ATP and ADP are released from the storage pool while the metabolic pool remains intact (9). Holmsen *et al.* (9) further showed that, on incubation with radioactive precursors, e.g., adenine, the metabolic pool can be labelled *in vitro* but the nonmetabolic remains unlabelled. When PRP, which had been previously incubated with 8-^{14}C-adenine, was filtered on Sepharose 2B using albumin–buffer eluting agent, radioactivity eluted in both platelet and plasma fractions (peaks A and B, Fig. 1). Chromatographic analysis of the plasma fraction (B, Fig. 1) revealed, in addition to unincorporated adenine, radioactive adenosine monophosphate (AMP), ATP, and ADP (Fig. 2). Radioactive nucleotides did not leach from stimultaneously conducted unfiltered PRP controls. Since radioactive adenine added to PPP remains unchanged for up to 5 hr (10), the radioactive plasma nucleotides could only have originated from the metabolic pool of the platelets. Extrac-

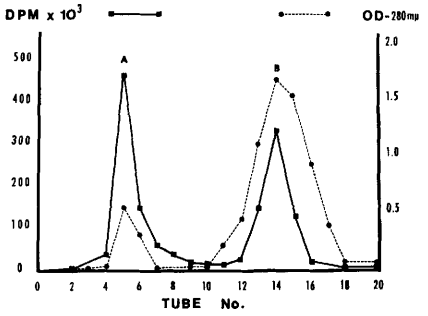

FIG. 1. The gel filtration pattern obtained with PRP after incubation with adenine-8-^{14}C (Platelets, peak A, Plasma, B).

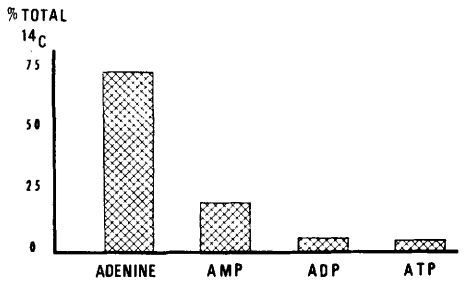

FIG. 2. ^{14}C-labeled adenine nucleotides present in the plasma area after gel filtration of PRP previously incubated with adenine-8-^{14}C.

tion and analysis of adenine nucleotides in the GFP platelets (A, Fig. 1) showed a tenfold increase in the specific activity of ADP, thus confirming that nucleotides were lost from both metabolic and nonmetabolic pools. The presence of these radioactive nucleotides in the supernatant plasma is indicative of damage occurring during passage of the platelet through the gel.

These studies support the conclusion that, although gel filtration is a simple and convenient method for separating platelets from plasma, technical refinements are necessary before this method can be considered appropriate for concentrating platelets for use in the clinical investigation of functional disorders.

Summary. Human platelets separated by gel filtration on Sepharose 2B were tested for aggregation to ADP, collagen, and epinephrine. Normal aggregation was observed only with ADP. Associated with the lack of functional response to collagen and epinephrine was the release of abnormally small amounts of ADP and ATP. Assay of platelets after gel filtration indicated that significant amounts of ADP and ATP had been lost. By labeling platelets prior to filtration it was shown that the released adenine nucleotides came from both the metabolically active and the nonmetabolically active pool.

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