2,3-Diphosphoglycerate: A Physiological Inhibitor of Platelet Aggregation

Abstract. *2,3-Diphosphoglycerate (2,3-DPG) may inhibit the platelet release reaction and the irreversible aggregation of human blood platelets induced by adenosine diphosphate, epinephrine, or norepinephrine. The effects of 2,3-DPG on platelet aggregation were more pronounced in cases with low hematocrit (< 30 percent). Dipyridamole and vincaminor potentiated the antiaggregating effect of 2,3-DPG. Erythrocytes (10^3 to 10^4 per microliter) exhibited a similar antiaggregating effect, especially when secured from anemic patients.*

2,3-Diphosphoglycerate (2,3-DPG), an organic phosphate of the erythrocytes, has been shown to influence the affinity of hemoglobin for oxygen (*1*, *2*). The concentration of 2,3-DPG in erythrocytes is relatively high [about 4 times that of adenosine triphasphate (ATP) and 20 times that of adenosine diphosphate (ADP)] although it is not a useful pathway for energy production (*3*).

A remarkable series of studies has demonstrated that in various types of hypoxemias the 2,3-DPG concentration in erythrocytes increases, presumably as a compensatory mechanism to assist oxygen delivery (*4*). Decreased affinity of hemoglobin for oxygen and a rise in 2,3-DPG within the red cell has been recorded in various types of hypoxia (*4*), such as that associated with (i) living in high altitudes, (ii) pulmonary insufficiency, (iii) cardiac right-to-left shunt, (iv) congestive heart failure, and (v) severe anemia of any type, including hemolytic anemias. Decreased oxygen affinity and a rise in 2,3-DPG has also been recorded in hyperthyroid subjects and in normal subjects after the administration of thyroid hormone (*5*), in patients with chronic uremia (*6*), in normal subjects after the administration of testosterone (*7*), and in some other conditions (*7*). It has been shown that in anemias platelet adhesiveness to glass is decreased, a phenomenon attributed by Hellem *et al.* (*8*) to the overall decrease in erythrocyte ADP. Adenosine diphosphate, thrombin, and collagen are generally considered as the physiological agents which trigger platelet aggregation or adhesion (or both) in hemostasis as well as in thrombus formation. Although it is not yet well known how ADP induces platelet aggregation, it has been shown that in vitro exogenous ADP (i) aggregates platelets and (ii) activates the platelets to release the endogenous ADP, which in turn stabilizes the initial platelet aggregate.

In severe anemia the 2,3-DPG concentration in the erythrocytes is elevated (*4*) and platelet aggregation or adhesion is decreased (*8*), whereas in polycythemia the 2,3-DPG concentration in the erythrocytes is low (*9*) and thrombotic phenomena are not uncommon. This suggests that 2,3-DPG not only decreases the hemoglobin oxygen affinity in erythrocytes but also inhibits platelet aggregation and thus plays a role in maintaining hemostatic homeostasis.

We studied the in vitro effects of 2,3-DPG on platelet aggregation (*10*) by using a dual-channel Payton aggregometer. Citrated platelet-rich plasma (PRP) with an average platelet count of 400,000 per microliter was secured by a silicone technique (*11*) from blood drawn from healthy individuals of both sexes and from patients with posthemorrhagic anemia (hematocrit < 30 percent). 2,3-Diphosphoglycerate was dissolved in normal saline and added to PRP at a final concentration of 20 to 200 μM (*12*), 30 seconds before the addition of ADP (final concentration 0.5 to 2.0 μM), epinephrine (final concentration 2 to 5 μM), or norepinephrine (final concentration 2 to 5 μM). Isotonic saline was substituted for 2,3-DPG in the control experiments.

Figure 1a shows a typical recording obtained when saline plus ADP was added to normal PRP. At $10^{-6} M$, ADP induced a biphasic curve of platelet aggregation, and complete aggregation was obtained in 2½ minutes. However, adding 200 μM 2,3-DPG plus ADP delayed the second phase, and complete aggregation was obtained after 5½ minutes. Similar results were obtained with 200 μM 2,3-DPG plus epinephrine or norepinephrine (Fig. 1, b and c).

Since the concentration of 2,3-DPG in erythrocytes is significantly increased in anemias or hypoxia (*4*) we studied the antiaggregating effect of 2,3-DPG in PRP secured from patients with acute posthemorrhagic anemia. A typical recording obtained with PRP from a 7-day-old case of gastrorrhagia with hematocrit of 30 percent is shown in

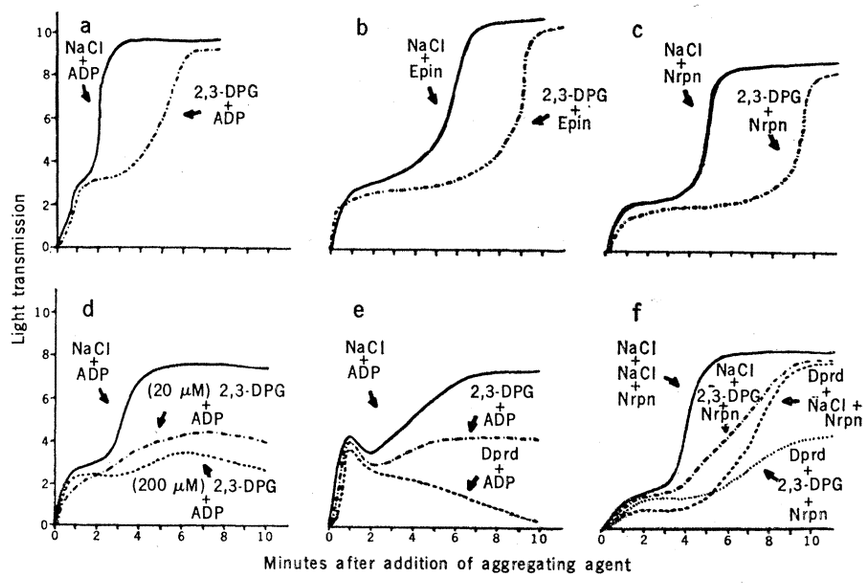

Fig. 1. Inhibition by 2,3-DPG of platelet aggregation induced by (a) 0.5 μM ADP, (b) 2 μM epinephrine, (c) 2 μM norepinephrine, (d) 0.5 μM ADP, (e) 1 μM ADP, and (f) 5 μM norepinephrine. Aggregation is indicated by increased light transmission through stirred (900 rev/min) platelet-rich plasma (PRP) at 37°C. Normal saline (NaCl, 0.05 ml), 2,3-DPG (0.05 ml), or dipyridamole (*Dprd*, 0.05 ml) was incubated with PRP (0.90 ml) at 37°C for 30 seconds before the addition of ADP (0.05 ml), epinephrine (*Epin*, 0.05 ml), or norepinephrine (*Nrpn*, 0.05 ml). In (f) the final concentrations of 2,3-DPG (0.025 ml) and dipyridamole (0.025 ml) were the same as in the other experiments.

Fig. 1d; in this case even 20 μM 2,3-DPG exerted a more pronounced inhibitory effect on ADP-induced platelet aggregation than 200 μM 2,3-DPG did with PRP from nonanemic individuals (Fig. 1a).

To determine whether the delay of the second phase observed after the addition of 2,3-DPG was due to its inhibition of the platelet release reaction, platelet factor-3 activation was measured by the shortening of the Russell viper venom (Stypven) clotting time of PRP according to the method of Zucker and Peterson (*13*). Figure 2 and Table 1 show that the addition of 2,3-DPG inhibited platelet factor-3 activation by ADP, epinephrine, or norepinephrine compared with their respective saline controls. Zachara (*14*) showed that the decomposition of high-energy phosphate compounds (mainly ATP, guanosine triphosphate, and 2,3-DPG) was much slower in solutions containing adenosine plus dipyridamole. The latter compound has been also shown (*15*) to inhibit in vitro ADP-induced platelet aggregation, but is without effect on aggregation produced by norepinephrine. Figure 1e shows the effects of 2,3-DPG (200 μmole/ml) and dipyridamole (100 μg/ml) on ADP-induced platelet aggregation in a 10-day-old case of gastrorrhagia with 36 percent hematocrit. Again the 2,3-DPG inhibition is pronounced, whereas dipyridamole induces complete deaggregation.

In view of the protective effect of dipyridamole on 2,3-DPG it is not improbable that the antiaggregating effect of dipyridamole is at least partially mediated by 2,3-DPG. Using norepinephrine as the aggregating agent, we showed that 2,3-DPG plus dipyridamole exerted a synergistic rather than an additive inhibitory effect on plaetelet aggregation (Fig. 1f). Similar results were obtained with 2,3-DPG plus vincaminor (*16*), a potent inhibitor of the second phase of platelet aggregation (*17*). The same synergism was also noted when other aggregating agents (ADP or epinephrine) were substituted for norepinephrine. The fact that the inhibitory effect of dipyridamole and vincaminor was potentiated by 2,3-DPG suggests that other compounds (such as aspirin or indomethacin) which have been shown to inhibit platelet aggregation may exert their inhibitory effect through 2,3-DPG.

These data support the hypothesis that 2,3-DPG is an inhibitor or a mediator of inhibition of platelet aggregation. Whether it does this by an enzymatic pathway involving direct or indirect increase of platelet cyclic adenosine monophosphate, by inhibition of prostaglandin E_2 or LASS (labile aggregation-stimulating substance) (*18*) formation, or otherwise has yet to be established.

Table 1. Effect of 2,3-DPG on platelet factor-3 activation. Results are expressed as percentage changes in the reciprocals of the clotting times with the samples incubated for 2 and 5 minutes compared with the 0-minute samples.

Sample	Percentage change in samples incubated for		
	0 min	2 min	5 min
ADP +			
Saline	100	103	133
2,3-DPG	100	108	106
Epinephrine +			
Saline	100	108	120
2,3-DPG	100	105	98
Norepinephrine +			
Saline	100	100	113
2,3-DPG	100	93	89

This hypothesis may explain the relation which apparently exists between a high 2,3-DPG concentration in erythrocytes and a low incidence of thrombosis or disseminated intravascular coagulation (DIC) in hypochromic anemias, sickle cell anemias, chronic nephritis, and so forth. It is of interest that in sickling crisis the majority of cases develop isolated microthrombi and only a few develop DIC. The high 2,3-DPG concentration probably prevents massive platelet aggregation in cases when large quantities of ADP and 2,3-DPG are simultaneously released in plasma from the hemolyzed erythrocytes.

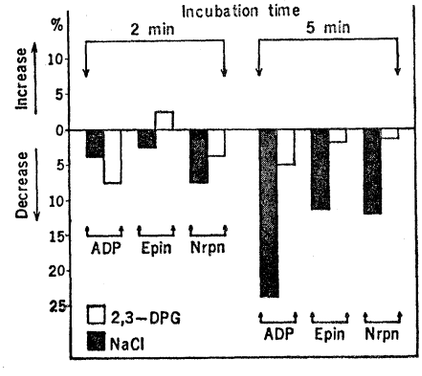

Fig. 2. Inhibition by 2,3-DPG of the induction of platelet factor-3 release by ADP (0.5 μM), epinephrine (*Epin*, 2 μM), or norepinephrine (*Nrpn*, 2 μM). Results are expressed as percentage decrease of the Russell viper venom clotting time of samples incubated for 2 and 5 minutes compared with the corresponding 0-minute sample.

If 2,3-DPG is indeed a physiologic inhibitor of platelet aggregation, then platelet aggregation should be affected by the number of erythrocytes present in PRP. We found that when the number of erythrocytes in PRP was increased two- to tenfold (from 2,000 μl^{-1} to 4,000 to 20,000 μl^{-1}) the rate and extent of platelet aggregation were strikingly inhibited. Furthermore, erythrocytes (5000 μl^{-1}) from anemic persons (with hematocrit < 30 percent and presumably a high 2,3-DPG concentration) exhibited a more pronounced inhibitory effect on PRP secured from healthy volunteers than did the same number of erythrocytes obtained from orthochromic normocytic persons.

It is interesting to speculate that in vitro platelet aggregation is related to the metabolic condition of the erythrocytes or platelets. The validity of this assumption is crucial in evaluating the importance of 2,3-DPG variation in maintaining hemostatic homeostasis. If this proves to be correct, then there is another good reason (apart from altering hemoglobin oxygen affinity) for developing methods or agents to elevate 2,3-DPG concentrations in erythrocytes. Such agents could be of great clinical usefulness in the treatment of thromboembolic diseases.

SOTIRIOS G. IATRIDIS
First Department of Internal Medicine, University of Athens School of Medicine, Vassilefs Pavlos Hospital, Athens 609, Greece

PANAYOTIS G. IATRIDIS
Department of Physiology, Northwest Center for Medical Education, Indiana University School of Medicine, Gary 46408

SOFIA G. MARKIDOU
Department of Physiology, University of Athens School of Medicine

BARTH H. RAGATZ
Department of Biochemistry, Indiana University School of Medicine

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11. Blood was routinely drawn from a vein in the arm with disposable plastic syringes. Platelet-rich plasma was obtained by centrifugation of 40 ml of blood plus 4 ml of 3.2 percent trisodium citrate in a plastic tube at 4°C for 10 minutes at 500 rev/min (130*g*). Usually 12 to 15 ml of PRP was pipetted off and the remaining plasma and blood were centrifuged for 15 minutes at 5000 rev/min (1500*g*) at 4°C to secure platelet-poor plasma. The plasmas were kept in an ice bath at 4°C and tested as soon as possible.
12. The concentration of 2,3-DPG used to demonstrate its inhibitory effect on aggregation (20 to 200 μM) is approximately 5 to 50 times greater than that detected in erythrocytes (*3*). However, in some experiments we have observed a significant inhibitory effect with 5 to 20 μM 2,3-DPG. Furthermore, local concentrations of 2,3-DPG released from erythrocytes in vivo and closely adjacent to the platelets may be considerably higher than those used in our in vitro experiments. In addition, in anemias and hypoxias 2,3-DPG concentrations are significantly increased (*7*).
13. Platelet-rich plasma was placed in the aggregometer; either isotonic saline or 2,3-DPG plus ADP, epinephrine, or norepinephrine were added; and the solutions were incubated for 0, 2, or 5 minutes. After the incubations, 0.1-ml portions were removed and pipetted into test tubes containing 0.1 ml of 25 mM calcium chloride and 0.1 ml of a solution consisting of 10 μg of Russell viper venom (Stypven of Burroughs Wellcome) per milliliter of imidazole buffered saline. The clotting times of these mixtures were measured and the results were expressed (Fig. 2) as the percentage decrease of the Russell viper venom time of the samples incubated for 2 and 5 minutes compared to the sample incubated for 0 minute [M. B. Zucker and J. Peterson, *Proc. Soc. Exp. Biol. Med.* **127**, 547 (1968)]. In Table 1 the results are expressed as the percentage changes of the reciprocals of the clotting times for the 2- and 5-minute samples compared with the corresponding 0-minute sample.
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Collagenase Production by Lymphokine-Activated Macrophages

Abstract. *Macrophages incubated with products (lymphokines) secreted by stimulated spleen cells produced collagenase. Active lymphokines were obtained both from mitogen- and antigen-stimulated lymphocytes. These observations suggest that the degradation of collagen in chronic inflammatory lesions may be caused by macrophage collagenase.*

Inflammatory lesions characterized by an infiltrate of mononuclear cells are often destructive to the surrounding connective tissue. The predominance of lymphocytes and macrophages in such a lesion suggests that they may have a central role in the degradation of connective tissue. Macrophages of alveolar origin have been shown to contain collagenase (*1*). However, macrophages from peritoneal exudates lack collagenase, but will secrete the enzyme when activated in culture by endotoxin (*2*). Since activated lymphocyte products are capable of stimulating certain functions of macrophages (*3*), we have tested the ability of soluble products (lymphokines) produced by mitogen- or antigen-activated lymphocytes to induce macrophages to produce collagenase.

Macrophages were obtained from the peritoneal cavity of guinea pigs 4 days after the injection of 20 ml of sterile mineral oil (Drakeol, Pennsylvania Refining). Dulbecco Vogt's medium (10 ml; NIH) containing 4×10^6 to 5×10^6 washed cells per milliliter was placed in plastic culture flasks (75 cm^2; Falcon). After incubation for 4 hours at 37°C, the nonadherent cells were removed by several washings with media. The remaining cells were 94 to 96 percent macrophages, as judged by their appearance and by the ability of cells to phagocytize latex particles, and 4 to 6 percent lymphocytes.

Soluble products produced by activated lymphoid cells were obtained as follows. Guinea pigs were immunized with 50 μg of dinitrophenylated ovalbumin (DNP-OA) in complete Freund's adjuvant by injection in the footpads. Two weeks later the spleens were excised from these animals, and cultures of splenocytes were prepared (*4*). The lymphoid cells (5×10^6 cells per milliliter) were suspended in serum-free RPMI 1640 medium (Grand Island Biological) or Dulbecco Vogt's medium and cultured in the presence or absence of DNP-OA (10 μg/ml), DNP–bovine serum albumin (10 μg/ml), or concanavalin A (1 μg/ml) (Calbiochem). After 24 hours, the cells were removed from the media by centrifugation, and the supernatants were dialyzed against distilled water and lyophilized. Antigen or mitogen was added to nonstimulated spleen cell cultures just prior to centrifugation, which served as a control. These products from the lymphoid cell cultures were added to the macrophage cultures at a concentration of 50 μg/ml. Media from the macrophage cultures were harvested daily and dialyzed against 0.03 mM tris (pH 7.5) and lyophilized.

The media (20 ml) in which macrophages were cultured were concentrated and assayed for collagenase with reconstituted collagen fibrils biosynthetically labeled with [^{14}C]glycine (*5*). Under physiological conditions cleavage of the helical portion of the collagen molecule is limited to collagenase. Trypsin (0.01 percent) was added to certain collagen substrates to obtain a measure of the substrate that could be solubilized without cleavage of the helical portion of the molecule. The reaction mixtures were incubated at 35°C for 16 hours, and the collagenase activity was then determined by measuring the labeled material in the supernatant.

Since we have observed that peritoneal macrophages produce collagenase after incubation with endotoxin (*2*), it was of interest to determine whether products from activated lymphocytes which stimulate various macrophage functions could also enhance collagenase synthesis. Indeed, when products from spleen cells stimulated with concanavalin A were added to macrophage cultures, significant collagenase activity was detected within 48 hours (Fig. 1). No collagenase activity was detected in media obtained from stimulated or control spleen cells. When the products produced by concanavalin A–stimulated cultures were added to macrophages in culture, collagenase activity was increased after a 1-day lag period. Essentially all the collagenase activity was present in the media. No increase in activity was observed in macrophage cultures receiving products from unstimulated spleen cells. In addition, products from supernatants of control spleen cells to which concanavalin A was added at the termination of culture did not induce collagenase release by the macrophages (Fig. 1), nor did they contain collagenase activity. In contrast to earlier studies in which lymphokine enhancement of macrophage function was not significant for 3 days (*3*), macrophage collagenase activity was maximum at 48 hours.