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HIGH ENERGY NUCLEOTIDE-INDUCED PULMONARY HYPERTENSION*

by

Peter Jokl
B.A. Yale University 1964

A thesis submitted to the Faculty of the Yale University School of
Medicine in candidacy for the Degree of

Doctor of Medicine

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DEDICATION

To Marion whose sincere interest
and guidance made this thesis
possible.

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Abstract

Adenosine di-phosphate elevates pulmonary arterial blood pressure more effectively, more consistently, and for longer periods than other high energy nucleotides that are endowed with similar hypertensive powers. No support could be found for the theory that aggregation of platelets and formation of wedge-thrombi are responsible for the ADP-induced rise of blood pressure.

Introduction

Pulmonary blood pressure is not constant. Physiological fluctuations accompany rest, exercise, and acute as well as chronic exposure to altitude.³ Because pulmonary artery pressure was known to be elevated at high altitudes,⁴ the effects of acute and chronic exposure to high altitudes have been extensively analyzed.^{5,6,7}

The present study originated from observations by Grover, et al., that pulmonary artery pressure in human subjects and in cattle living at altitudes of 7,000 - 10,000 feet above sea level was elevated.^{5,8} Furthermore, the investigators found that prolonged exposure to altitude sensitized the pulmonary arteries towards hypertensive agents.⁹

Since 1929, reports have appeared in the literature stating that the intravenous injection of the high energy adenosine nucleotides alter pulmonary artery pressure. However, precise information regarding the mode of action of high energy nucleotides was lacking. We therefore analyzed the pulmonary pressure effects of several high energy nucleotides. The experiment included measurements of pulmonary and aortic pressure in animals living at near-sea-level,* as well as on animals who had been adapted to simulated altitudes of 11,000 feet. Since Gaarder had demonstrated that ADP aggregates platelets in vitro,¹⁰ I postulated that the possible mechanism of the nucleotide induced rise in pulmonary artery pressure was due to platelet aggregates blocking part of the capillary outflow of the pulmonary bed. If a large number of platelets were sequestered in the pulmonary vessels, a significant

*Lexington, Kentucky (955 feet above sea level)

reduction in the arterial platelet count could be expected. Therefore, serial arterial platelet counts were undertaken during the injection of two nucleotides adenosine diphosphate (ADP) and guanosine diphosphate (GDP). Both these compounds were known to elevate pulmonary artery pressure. Finally, the literature relating to chemistry, physiology, pharmacology, and toxicology of the compounds was surveyed.

Historical Remarks

I. Nucleotides

In 1847, Justus von Liebig discovered inosinic acid, a metabolic precursor of purines.¹¹ In 1871, Friedrich Miescher identified nucleic acid. He isolated the nuclear components of white blood cells, using as his source of material cells extracted from discarded surgical bandages. By digesting these pus cells with pepsin and hydrochloric acid and then shaking these cells with ether, he obtained a leucocyte nuclei concentrate he called "nuclein".¹² Picard, a co-worker of Miescher, treated salmon sperm using the above method and identified the purine base guanine.¹³ In 1885, Alfred Kossel isolated adenine from acid hydrolysates of beef pancreas nucleic acid. In 1894, Kossel succeeded in purifying cytosine from calf thymus-nucleic acid.¹⁴ In 1900, Ascoli obtained uracil following the acid hydrolysis of yeast-nucleic acid.¹⁵ The isolation of muscle adenylic acid (AMP) was accomplished in 1927 by Embden and Zimmerman.¹⁶ Two years later, adenosine triphosphate was purified by Lohmann, a co-worker of Otto Meyerhof and by Fiske and Subbarow.^{17,18}

The metabolic, biochemical and biophysical effects of high energy nucleotides in vivo was studied by Lohmann, Meyerhoff, Szent Gyorgyi, A. F. and H. E. Huxley, and Fritz Lipmann.^{19,20,21,22}

The following is a summary of those reactions about which knowledge is necessary for the understanding of the chemical nature of the compounds that were used in our experiments. When purine or pyrimidine bases are combined with sugar, nucleosides are formed. If the nucleoside reacts with phosphoric acid, a nucleotide is formed (a phosphorylated nucleoside is

called a nucleotide). Two types of nucleotides are contained in nucleic acids: those nucleic acids containing the sugar moiety D-ribose are called ribonucleic acids (RNA) and those containing 2-deoxy-ribofuranose are called deoxy ribonucleic acids (DNA). Tissue also contains nucleotides that are not combined in nucleic acids. Most important of these are the adenosine phosphates which act as an energy source for muscle contraction and as an activator of enzymes and of metabolic precursors. Thus, AMP is of importance in activation of phosphorylase. Likewise, ADP and ATP play an essential part in oxidative phosphorylation and thus represent a source of energy. Many reactions such as the incorporation of sulfate into ester linkages in compounds like sulfated mucopolysaccharides require the preliminary formation of active sulfate with ATP.

The biological importance of other nucleotides used in this study are as follows. Inosine is a metabolic precursor of adenine and guanine. It can be synthesized from adenosine by deamination with nitric acid in vitro. A guanine derivative is required for the oxidation of ketoglutaric acid to succinate. This is involved in oxidative phosphorylation with transfer of phosphate to guanosine diphosphate to form guanosine triphosphate which is a source of stored energy. Cytidine triphosphate (CTP) is the only nucleotide effective in the in vitro mitochondrial system for the biosynthesis of lecithin.²⁴

II. Platelets*

Blood platelets were first identified in 1842 by Donnè who

*The term "Blut-plättchen" (tr. blood platelets) was introduced by Bizzozero in 1881. Osler in 1883 translated Bizzozero's work and thus introduced the word "platelets" into the English medical terminology.²⁵

called them "globulins", and believed they were derived from "lymph".²⁶ In 1874, Osler referred to "organisms" in the blood of rats and man which he initially thought were bacteria.²⁷ However, his illustrations leave no doubt that these "organisms" were platelets. In addition, Osler²⁸ observed that these particles formed granular aggregates in coagulating blood. Hayem, who corroborated Osler's findings, thought that the "organisms" were precursors of red cells and therefore called them "hematoblasts".²⁶ In 1906 Wright correctly said that platelets were fragments of the cytoplasm of giant cells of bone marrow.²⁹ These cells were described as early as 1869 by Bizzozero and named megakaryocytes by Howell in 1890.³⁰

The first attempt to count platelets was made by Riess in 1872, who associated changes in the platelet count with various diseases.³¹ In 1878, Hayem reported normal platelet counts on human subjects (317,000 to 200,000) that do not differ significantly from those accepted today.²⁶ In 1882, Hayem suggested that platelet thrombi might play an important role in the arrest of bleeding and that a decrease in numbers or absence of platelets would cause a disruption of the hemostatic process.²⁶ Duke (1912) found that a close correlation existed between the number of platelets in the blood and bleeding time.³²

In 1956, Born discovered that platelets which had been known to contain histamine, 5-hydroxytryptamine, and adrenaline, also had large amounts of adenosine triphosphate (ATP) (up to 4.5 μ moles ATP/g in platelets of pigs, and 1.9 μ moles/g (wet weight) in human platelets).³³ Furthermore, he observed that ATP rapidly disappeared during clotting and that after coagulation

of whole blood, ATP could no longer be recovered from serum.³⁷

In 1960, Hellem described a fraction in red blood cells which he called "Factor R" which was "acidic, dialysable and heat stable." Factor R increased the adhesiveness of platelets. This adhesiveness was measured by noting the increase in the number of platelets in platelet-rich plasma which adhere to a glass column before and after they were treated with "Factor R". Intravenous injection into rabbits of "Factor R" was followed by a short-lasting decrease of the platelet count. This phenomenon was felt to be due to platelets being sequestered in the lungs and in other organs.³⁵

In 1961, Gaarder showed that "Factor R" was adenosine diphosphate (ADP). He tested other nucleotides for their effect on the adhesiveness of human platelets and found that only deoxyadenosine diphosphate was active.¹⁰ In the same year, Born noted that ADP aggregated platelets in vitro.³⁶

Aggregation of platelets by ADP was found to be a highly specific process. Substances closely related to ADP such as adenosine monophosphate (AMP), adenosine and 2 chloroadenosine acted as competitive inhibitors of ADP induced platelet aggregation.³⁷

In addition to the above effects, the concentration of platelets in blood was found to fluctuate markedly with several physiological stimuli. For example, intensive physical exercise between one and ten minutes duration might double the number of platelets.^{38,39} Recently, changes in the peripheral blood platelet concentration have been noted in animals subjected to rapidly changing atmospheric pressure.⁴⁰

Review of Literature

In 1929, Drury and Szent-Gyorgyi performed the first experiments in which the effects of nucleotides upon the pulmonary circulation were documented.⁴¹ Since that time, reports concerning both the action and the mechanism of the nucleotide induced pulmonary artery pressure changes have varied widely.

A. N. Drury and Szent Gyorgyi isolated an extract from heart muscle and identified it as adenylic acid (AMP). "When injected intravenously into the whole animal, it disturbed the cardiac rhythm in a constant and definite manner."⁴¹ In the isolated heart-lung preparation, AMP caused an increase in pulmonary artery pressure believed to be due to a left-to-right shunt caused by dilation of coronary vessels. In 1931, Bennet and Drury who perfused isolated rabbit lungs with Ringer's solution noted that addition of 2-4 mg of adenylic acid (AMP) led to a "slight increase in pulmonary artery pressure."⁴² However, the extract used in these studies was not pure. Being a muscle extract, it presumably contained significant amounts of adenosine diphosphate. In 1948, Emmelin and Feldberg observed that injection of 0.2 to 0.4 mg adenosine triphosphate into the right auricle or into the jugular vein of cats produced "a tremendous swelling of the pulmonary artery and the whole right heart, ventricle and auricle." At the same time, pulmonary artery pressure rose from 20 to 80 mm Hg.⁴³ In 1933, Gaddum and Holtz reported that small doses of adenosine di- and tri-phosphate caused dilation of the pulmonary vessels in cats, while a vaso-constrictive effect became evident after injection of larger amounts of the same compound. Injection of 0.005

mg of adenosine and adenosine-monophosphate raised pulmonary artery pressure, though not as much as corresponding amounts of adenosine diphosphate.⁴⁴

In 1961, Gordon studied the effect of i.v. injections of adenosine mono-, di-, and tri-phosphate on right ventricular pressure of rats. Thirty $\mu\text{g}/\text{kg}$ caused a very small rise of 1 or 2 mm Hg.⁴⁵

Two reports of the effect of adenosine compounds upon human subjects were available. Neither of them, however, contained measurements of pulmonary artery pressure. Davies injected ATP intravenously and intra-arterially. Some of his subjects "experienced peculiar sensations in the chest, reported difficulty in breathing, and had to cough during the injections," while others stated that breathing became "easier!"⁴⁶ The other report was by Davey who noted that 5 mg of ADP produced palpitation, light-headedness, tightness of the chest, and tachycardia.⁴⁷

In experiments with cats, Stoner and Green found that intravenous injection of adenine compounds led to a rise in pulmonary artery pressure; that ADP and ATP were equally effective pressor substances and that AMP caused but 1/3 and ITP 1/10 the magnitude of the response of ATP. Adenosine, guanylic acid (GMP) and cytidylic acid (CMP) were found to be inactive. The observed increases in pulmonary artery pressure were interpreted as being due to vaso-constriction.⁴⁸

The following is a summary of extracellular reactions to adenosine compounds so far demonstrated:

1. Dilation of peripheral and splanchnic vessels (ATP, AMP)^(41,43,48,49,50)

2. Inhibition of respiratory center (ATP)(48)
3. Rise in venous pressure (ATP)(48)
4. Fall in arterial pressure (ATP, ADP, AMP)(48,51,52,53)
5. Dilatation of coronary arteries (AMP) (48,51,54)
6. Decreased heart rate (ATP) (48,51)
7. Dilatation - vaso-constriction of renal vessels (ATP) (48)
8. Contraction of uterus (ATP) (48,51)
9. Contraction of bladder (ATP) (48)
10. Dilatation of cerebral vasculature (55)
11. Aggregation of platelets (ADP) (10)

Although it is known that adenine nucleotides influence pulmonary arterial pressure, the results have been variable. Furthermore, the evidence contained in the literature failed to elucidate the effect on pulmonary artery pressure of other nucleotides, including those in the pyrimidine group. No conclusive proof was available concerning the mechanism by which those nucleotides, which were known to raise pulmonary artery pressure, exerted their influence.

Theoretical Considerations

Two possible mechanisms of nucleotide-induced pulmonary hypertension were considered by the author.

- 1) Increased blood flow through the lungs;
- 2) Increased resistance to the flow of blood in the pulmonary vascular bed.

I. Increased Flow

The possibility that a left to right shunt caused pulmonary hypertension following injection of nucleotides was suggested as early as 1929 by Drury and Szent Gyorgyi.³⁹ They observed that an injection of adenosine isolated from muscle caused a rise in pulmonary artery pressure to three times its pre-injection level. This effect was ascribed to dilation of the coronary vessels. However, it is likely that the muscle extract contained significant amounts of ADP which caused the pressure changes. Since flow in the pulmonary stream bed can be doubled with only slight increases in pulmonary artery pressure,⁵⁶ the shunting theory formulated by Drury and Szent Gyorgyi was not acceptable.

II. Increased Resistance

It seemed possible that the nucleotide induced effect upon the pulmonary artery pressure might be due to changes in tone of the pulmonary vasculature. The pulmonary vasculature reacts to a number of physiological and pharmacological stimuli which increase or decrease pulmonary artery pressure through vaso-constriction or vaso-dilation, respectively. Anoxia,

sympathetic nerve stimulation, 5-hydroxytryptamine, hypertonic solutions, bacterial toxins, histamine, epinephrine and levarterenol cause vaso-constriction. Aminophylline, papaverine, tolazoline, nitrates and acetylcholine produce vaso-dilation.^{57,58}

Gaddum and Holtz reported a series of experiments on the effects of the adenosine compounds on the pulmonary vasculature of isolated dog and cat lungs perfused at a constant flow rate with heparinized blood. By measuring the changes in volume of the isolated lungs, the authors concluded that adenosine, AMP, and ADP in small doses (0.1 mg) caused vasodilation and larger doses (0.25 mg) resulted in a "vasoconstrictive effect".⁴⁴

Emmelin and Feldberg (1948)⁴³ and Stoner and Green (1951)⁴⁸ concluded that ATP caused systemic hypotension and pulmonary hypertension. The systemic effect was thought to be secondary to pulmonary vaso-constriction. However, Gordon (1961) showed that the systemic depressor action of adenosine compounds in rats was independent of pulmonary vaso-constriction. Rats which responded to injection of AMP, ADP, and ATP with a marked decrease in systemic pressure showed no concomitant changes in pulmonary artery pressure. Gordon suggested that the decrease in systemic artery pressure was solely due to peripheral vaso-dilatation.⁴⁵

The possibility that the observed increase in pulmonary vascular resistance was secondary to mechanical obstruction was based on the following data found in the literature.

Born injected ADP i.v. into cats, and found a brief but dramatic decrease in the number of blood platelets. Of the four cats into which Born infused ADP, the systemic blood pressure remained unchanged in two. In

the other two, there was a brief pressure drop, followed by prompt re-establishment of pre-infusion levels. AMP and adenosine in molar concentrations equal to those of ADP elicited no change in the platelet count, but did cause a decrease in the systemic blood pressure. Thus, in some of Born's experiments, ADP reduced the platelet count without affecting blood pressure; in others AMP reduced blood pressure without affecting the platelet count.⁵⁹ The author concluded from this evidence that ADP affected the platelet count independent of changes in blood pressure.

Regoli and Clark subsequently showed that prior infusion of adenosine into rabbits followed by injection of ADP prevented the drop in platelets cause by ADP. They also demonstrated that the systemic vaso-dilator effects of adenosine and of ADP were independent of the changes in the platelet count of the blood.⁶⁰

Davey (1964) investigated the effect of ADP on chromium 51-labeled platelets in human subjects. He noted that intravenous doses of 2.5 - 20 mg ADP caused a significant reduction in arterial platelet count concomitant with an increase in radioactivity over the spleen and liver. When ADP was infused into the brachial artery, marked vaso-dilation took place in the injected extremity, unaccompanied by changes in the platelet count in blood taken from the same arm's brachial vein. Davey concluded that the reduction of platelets following administration of ADP into the systemic circulation was due to transient concentration of platelets in the splanchnic and pulmonary capillaries.⁴⁷

In 1964, Nordoy and Chandler autopsied the lungs of male albino rats twenty seconds after intravenous injection of ADP (25 mg/100gm of body weight).

Numerous platelet thrombi were found in pulmonary capillaries. In animals which had been given lesser doses of ADP, the number of thrombi seen was smaller. In animals given 1 mg ADP/100 gm and sacrificed sixty seconds later, no platelet thrombi were observed, while a "moderate" number of thrombi was seen in animals given 7 mg ADP/100 gm. Respiration was invariably affected. Following the administration of small doses of ADP, there was slowing of respiration, while high doses caused complete arrest of breathing lasting up to sixty seconds. As cause for this phenomenon, the author considered "sudden occlusion of pulmonary capillaries", since infusion of small-particulate matter (Sephadex) also elicited respiratory arrest.⁶¹ Finally, from clinical experience it was known that pulmonary artery pressure rises chronically in patients suffering from multiple pulmonary emboli.^{5,62} Considering the above data, it was hypothesized that nucleotide-induced pulmonary hypertension might be caused by the occlusion of pulmonary vessels by platelet aggregates. Moreover, if sufficient platelets were trapped in the lung, a decrease in arterial platelet counts should be expected.

Design of Study

Since chemically pure nucleotides had not been systematically evaluated nor pressor responses of the pulmonary arteries accurately measured, it was decided to conduct a series of experiments to elucidate the response of pulmonary arterial pressure to naturally occurring nucleotides. In planning the study, consideration was given to the appropriate statistical pre-requisites. The injections of nucleotides were given in random order and the significance of the data obtained was analyzed by paired T tests with associated probabilities between corresponding pairs of data.

After the pulmonary hypertensive effect of the high energy nucleotides had been observed,^{1,2} arterial platelet counts were made following the injection of two nucleotides found to cause pulmonary hypertension. These nucleotides were ADP which clumps platelets in vitro and GDP which does not. Blood for platelet counts was drawn during the measurements of the ADP-and GDP-induced pulmonary arterial pressure rise. A correlative evaluation of the two phenomena under scrutiny i.e. the change in pulmonary artery pressure and arterial platelet count could thus be done. The statistical design for the platelet study was essentially the same as that used for the analysis of the pressor responses.

Methodology

The experiments were conducted with calves (one to four months of age), weighing from fifty to seventy kilograms.* ** No sedative or tranquilizing drugs were given, since evidence was available showing marked alterations of circulation and respiration were caused by these agents.⁶³ The animals were catheterized in the usual manner.⁶⁴

After clipping and cleaning the left side of the neck, a 14 gauge needle was thrust into the external jugular vein and a 36 inch length of PE/90 Adams Co. Intramedic polyethylene, saline filled catheter was inserted. The catheter was now attached via a 3-way stop cock to a Statham pressure transducer which was connected with a Sanborn 151 recorder. The location of the catheter tip was monitored with the aid of the pressure records. The catheter was advanced manually. Pressure tracings of right ventricle and pulmonary artery were recorded. The position of the catheter was checked throughout each experiment and care taken that it was situated immediately beyond the pulmonary valves before the bifurcation. Using the 3-way stop cock, measured doses of high energy nucleotides were rapidly injected. The catheter was flushed with 3 cc of normal saline after each injection. The stop cock arrangement allowed the use of the same catheter for injections as well as for the recording of pressure. Recording of tracings thus had to be interrupted for not more than five seconds during injections.

In several experiments intra-aortic pressure was also studied.

Polyethylene tubing was introduced into the dorsal aorta which was punctured percutaneously in the eleventh left intercostal space with a No. 14 gauge needle. Pressure measurements were recorded at paper speeds of 1.00 mm/sec. Zero reference point in standing animals was a mid-chest position; in animals, lying on the side, a point half the width of the chest.

*Calves were used for the following reasons:

- 1) Calves have a conspicuously reactive pulmonary vascular bed, (65,66).
- 2) Calves are not disturbed by the experiment, e.g. they continue to eat while being catheterized.
- 3) Calves are large animals so that the taking of numerous blood samples presents no problem.
- 4) In calves the position of external jugular veins and aorta is such as to render percutaneous catheterization easy.
- 5) There are no difficulties in keeping calves in cages.

**Three calves were maintained in a low pressure chamber at a simulated altitude of 11,000 feet. The chamber was opened only for feeding (twice a day) and during the experiments.

The following nucleotides were supplied by P-L Biochemicals, Inc., Milwaukee, Wisconsin: the 5' tri-, di-, and mono-phosphate ribonucleotides of the inosine, guanosine, and adenine in the purine nucleotide series and cytidine and uridine of the pyrimidine nucleotides.

In the experiments in which the nucleotide effect on pulmonary arterial pressure was studied, 100 /ug of each of the above named compounds were dissolved in 5 cc of saline. In the experiments in which platelet counts and pulmonary artery pressure were monitored, equal molar amounts (2×10^{-7} moles) of ADP and GDP were dissolved in 5 cc of saline.* In each instance the total amounts were injected as a bolus dose. Blood samples for the platelet counts were withdrawn from the aorta. All injections (Saline, ADP and GDP) were given into the pulmonary artery in random order. Samples for platelet counts were withdrawn in accordance with the following time schedule: one preinjection sample, one sample 30 seconds after the injection of the test substance, followed by further withdrawals 1.5, 2.5, 3.5, 4.5, 10 and 15 minutes later. At that time, blood pressure levels had invariably returned to pre-experimental values.

Five cc "Vacutainers" containing 6 mg of EDTA were used to collect the blood. To prevent clumping of platelets in the polyethylene catheter, blood was allowed to flow at a slow rate. Whenever time intervals between withdrawals of up to five minutes following injections were called for, the polyethylene catheter was filled with heparin. Thirty seconds before collection of such samples, heparin was allowed to run out whereupon blood was

* 2×10^{-7} Moles represents 100 micrograms of ADP and 105 micrograms of GDP, both dosages causing significant increases in pulmonary artery pressure.

withdrawn at a slow, steady rate.

Platelets were counted by the phase contrast method as described by Brecher and Cronkite.⁶⁷ The significance of the obtained data was analyzed according to the paired T test. (For formulas, see Appendix.)

Results

Table 1 shows mean pulmonary artery pressures* and their standard deviations, pressure change after injection of nucleotides, P values, and duration of observed effects.

The triphosphate and diphosphate compounds of the purine bases adenosine (ATP, ADP), guanosine (GTP, GDP), and inosine (ITP, IDP) and the diphosphate compound of the pyrimidine uridine (UDP) elicited statistically significant pulmonary pressure responses. With the exception of UDP, all pyrimidine compounds that were tested and all monophosphate purine derivatives failed to engender significant pressure changes. Except for the guanosine group, the diphosphate compounds produced consistently greater rises of pulmonary pressure than did their triphosphate counterparts. Of the pyrimidine nucleotides, only uridine diphosphate led to significant changes in pulmonary pressure. CTP, CDP, CMP, UTP, and UMP were nonreactive. (Fig. 1 and 2). Individual examples of the effects of the nucleotides are detailed in Fig. 3 and 4. ADP was the most potent agent. Doses as small as 1.25 ug (0.23×10^{-8} moles) produced rises in pulmonary pressure by margins of greater than 5 mm Hg (Table 2).

As mentioned earlier, in several experiments pulmonary artery and aortic pressures were recorded simultaneously (Fig. 5). Whenever pulmonary pressure rose following the injection of 100mg of ADP, the systemic arterial

*Pulmonary artery pressures are represented throughout this report as averages of systolic and diastolic pressures. Mean values in the accompanying tables refer to arithmetic midpoints from individual experiments of the various series under analysis.

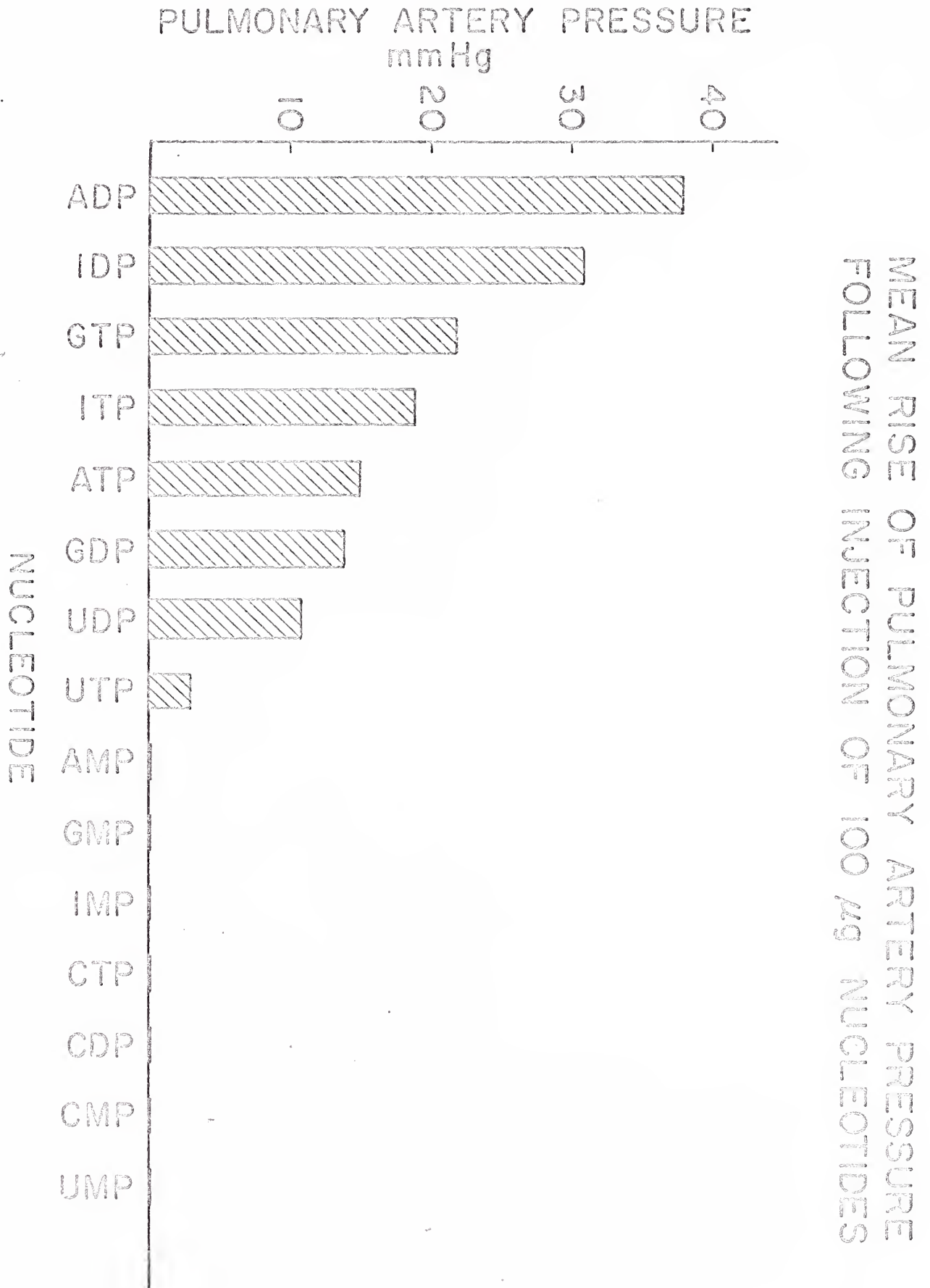
Table 1

Effect of injection of 100 ug of high energy nucleotides on pulmonary arterial pressure of calves (means, S.D., and P values).

Table 1.

Effects of injections of 100 ug of high energy nucleotides on pulmonary arterial pressure of calves (in mmHg)

Drug	Sample Size	Mean Pre-Exp. Values - S.D.	Mean Values (Max.) - S.D. After Injection	Pressure Change After Injection	P	Duration of Pressure Rise (in minutes)
Saline	19	21 +6.4	21 +6.2	0	0.42932	0.0
ATP	7	21 +7.0	36 +14.0	15	0.01799	4.7
ADP	7	22 +4.6	60 +11.0	38	0.0000	6.6
AMP	6	20 +5.4	21 +5.2	1	0.37628	0.0
GTP	6	20 +5.1	42 +17.6	22	0.01988	4.4
GDP	8	23 +5.7	37 +9.6	14	0.00107	5.3
GMP	6	21 +5.0	21 +5.0	0	0.50000	0.0
ITP	8	22 +5.7	41 +18.8	19	0.01587	3.4
IDP	8	21 +5.1	52 +17.8	31	0.00144	4.2
IMP	8	23 +4.8	22 +5.3	-1	0.38640	0.0
CTP	9	23 +5.6	23 +5.6	0	0.50000	0.0
CDP	7	22 +5.7	22 +5.7	0	0.5000	0.0
CMP	8	23 +5.8	23 +5.4	0	0.46484	0.0
UTP	8	19 +5.7	23 +8.1	3	0.15901	0.8
UDP	8	21 +6.5	32 +13.5	11	0.03960	1.3
UMP	8	20 +5.7	20 +5.7	0	0.50000	0.0



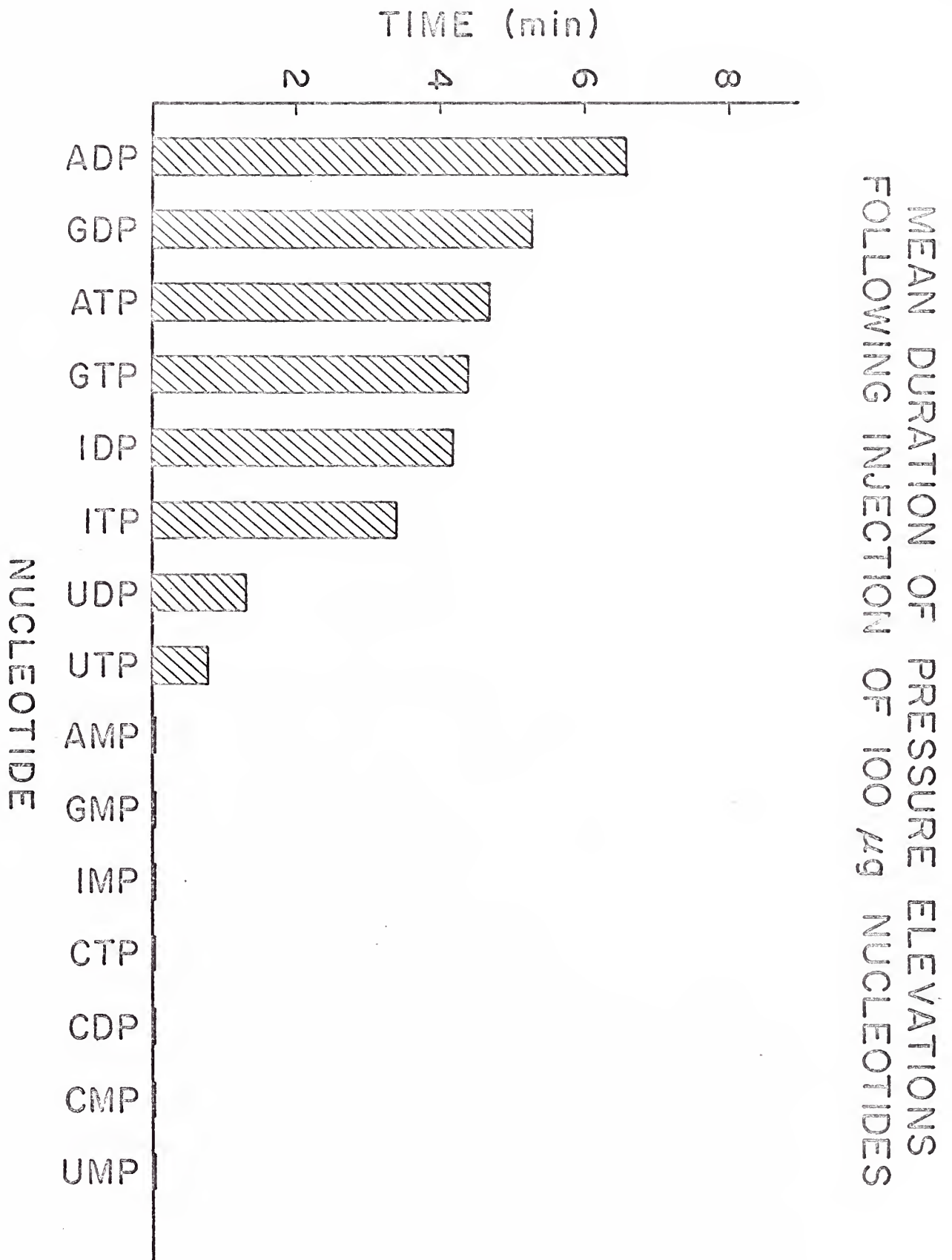


Figure 3

Effect of 100 μ g of adenosine nucleotides on pulmonary arterial pressure (AMP - adenosine monophosphate, ADP - adenosine diphosphate, ATP - adenosine triphosphate).*

*In fig. 3-7, results of representative individual experiments are depicted.

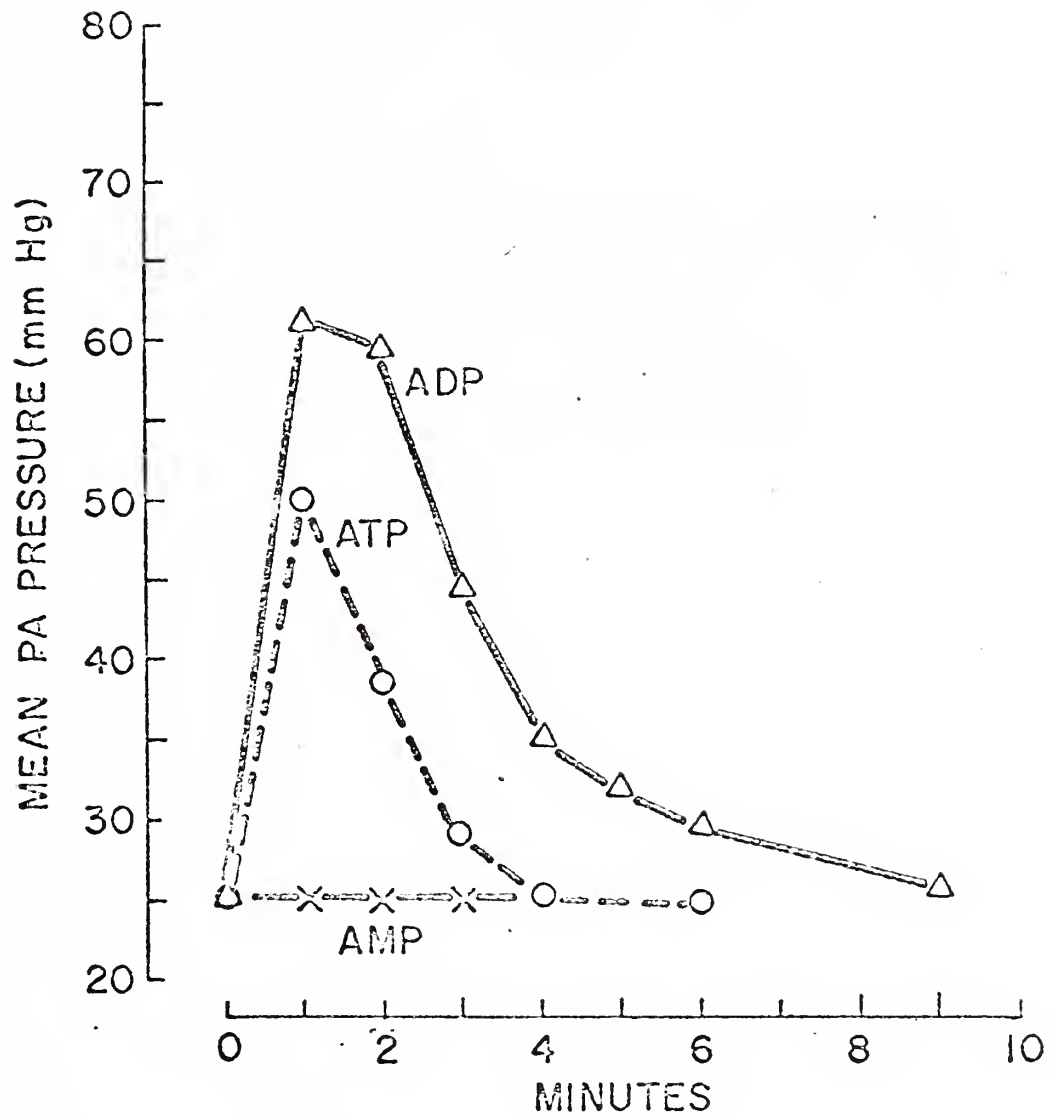


Figure 4

The effect of 100 μ g of a diphosphate purine nucleotide, adenosine diphosphate (ADP) and a diphosphate pyrimidine nucleotide, uridine diphosphate (UDP) on pulmonary artery pressure (ADP - adenosine diphosphate, UDP - uridine diphosphate).

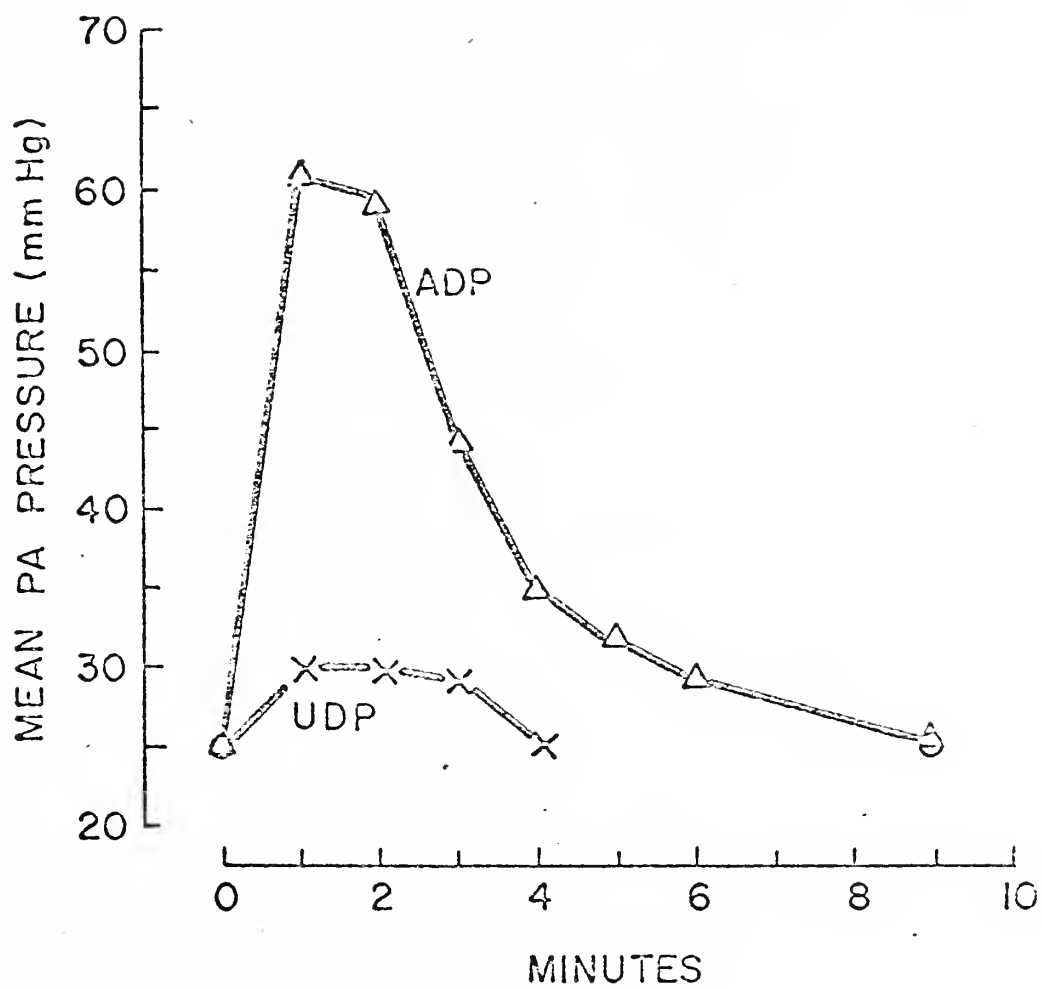


Table 2:

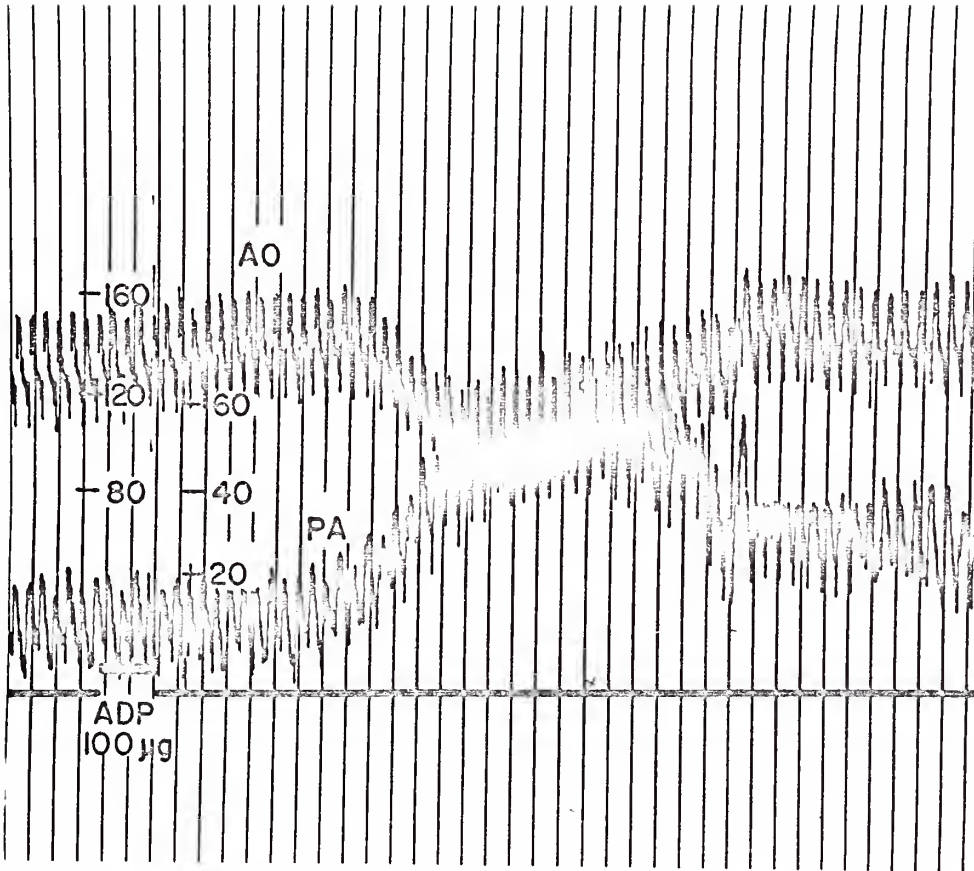
Minimum doses of nucleotides that increase pulmonary arterial pressure 5 mm Hg or more. (For identification of nucleotides, cp. p. 20)

MINIMUM EFFECTIVE DOSE

Nucleotide	Calf #11		Calf #12	
	<u>Micrograms</u>	<u>Moles</u>	<u>Micrograms</u>	<u>Moles</u>
ITP	50	8.4×10^{-8}	50	8.4×10^{-8}
IDP	25	5.1×10^{-8}	25	5.1×10^{-8}
UTP	50	8.5×10^{-8}	100	17×10^{-8}
UDP	25	5×10^{-8}	50	10×10^{-8}
GTP	25	4.1×10^{-8}	25	4.1×10^{-8}
GDP	50	10×10^{-8}	25	5×10^{-8}
ATP	25	4.1×10^{-8}	10	1.6×10^{-8}
ADP	5	1×10^{-8}	1.25	$.23 \times 10^{-8}$

Figure 5

Effect of 100 ug adenosine diphosphate (ADP) on pulmonary arterial (PA) and aortic (AO) pressures (Time intervals: 1 sec.). The vertical scale on the left shows aortic pressure, the scale next to it pulmonary arterial pressure.



pressure decreased, reaching its lowest level when the pulmonary pressure response reached its maximum. Aortic pressure then rapidly returned to its starting level while pulmonary arterial pressure remained elevated for several more minutes. The injection, at sealevel, of the nucleotide compounds into three high altitude (11,000 ft.) acclimatized calves showed significantly greater increases in pulmonary artery pressure than those observed in the low altitude calves (Table 3, Fig. 6).

Some of the altitude acclimatized calves developed prolonged pulmonary hypertension following injection of high energy nucleotides (Fig. 7). These findings are noteworthy since similar responses had previously been observed only in new born animals.⁶¹ Neither had it been known that altitude acclimatized calves several months of age are capable of pulmonary pressure responses of the kind under discussion, nor that high energy phosphates can trigger them. Repeated doses of the same nucleotides applied later on the same day caused pressure responses of shorter duration. (Fig. 7)

Since it was known that solutions of low pH induce vasoconstrictor responses in the pulmonary vasculature, the pH of all nucleotide solutions was measured. As can be seen from Table 4, there was no correlation between the pH of a nucleotide solution and the observed pulmonary pressure responses.⁶⁸

No significant changes in the number of platelets were noted at any time after the injection of saline, ADP, or GDP despite the changes in the pulmonary artery pressure (Fig. 8, Table 5 Appendix). The standard deviations of platelet counts from six calves were very great, extending from 14,800 to

184,300 (Table 6). This range was greater than that found by Brecher et al., but about the same as that reported by Born and Gaarder. In some animals, platelet counts dropped initially, concomitant with the beginning of rise in pulmonary artery pressure. However, no further parallelism between the two parameters under analysis was noted.

Table 3:

Effect of injection of 100 ug of high energy nucleotides on pulmonary arterial pressure of three calves which had lived since birth at a simulated altitude of 11,000 feet. Measurement taken at near sea-level altitude (in mm HG).

Drug	Mean Pre-Exp. Values	Mean Values (Max.) After Injection	Pressure Change After Injection	Duration of Pressure Rise (in minutes)
TP (5)	47	103	56	3
DP (3)	34	89	55	9
TP (5)	43	86	43	4
DP (1)	65	115	50	12

Numbers in parentheses are the numbers of observations. For comparison with corresponding pulmonary artery pressures recorded from animals living at near sea level throughout see Table 1.



Figure 6:

Response to ADP of pulmonary arterial pressure of a calf which had lived since birth at a simulated altitude of 11,000 feet (straight lines) and of a calf which lived at near-sea-level (dotted lines). The abscissa represents doses of ADP. Measurements taken at near sea-level altitudes.

Fig. 6.

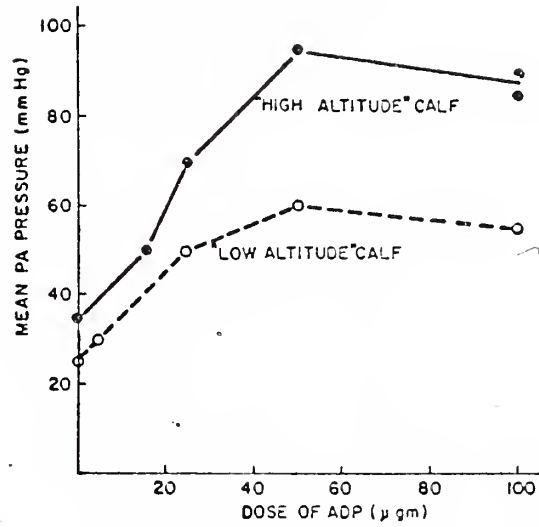


Figure 7:

Left side of diagram: Response of pulmonary artery pressure at near sea level altitude to 100 ug GDP in four months old calf which had lived since birth at a simulated altitude of 11,000 feet.

Right side: Responses of pulmonary artery pressure to 200 ug GTP injected five minutes after pressure had returned to normal level following foregoing experiment.

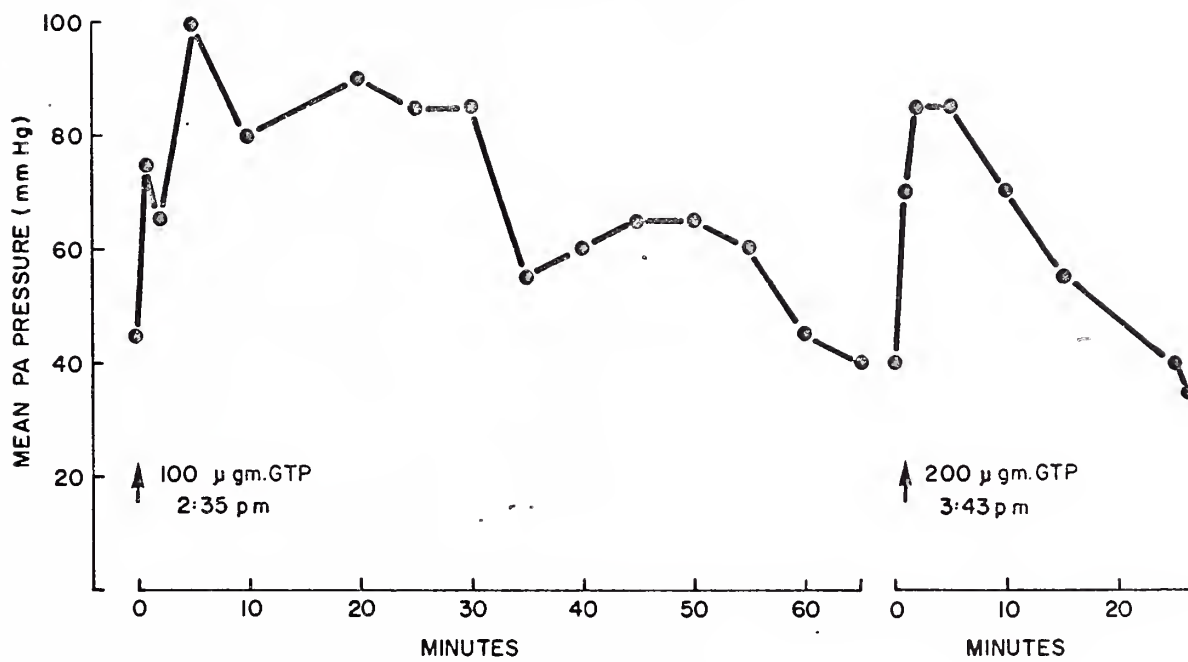


Table 4:

pH measurement of nucleotide solutions which were used in the experiments observing pulmonary pressor effects. There is no correlation between pH of the nucleotide solution and pulmonary pressor activity of nucleotide solution. (cp. table 1 & fig. 1,2.)

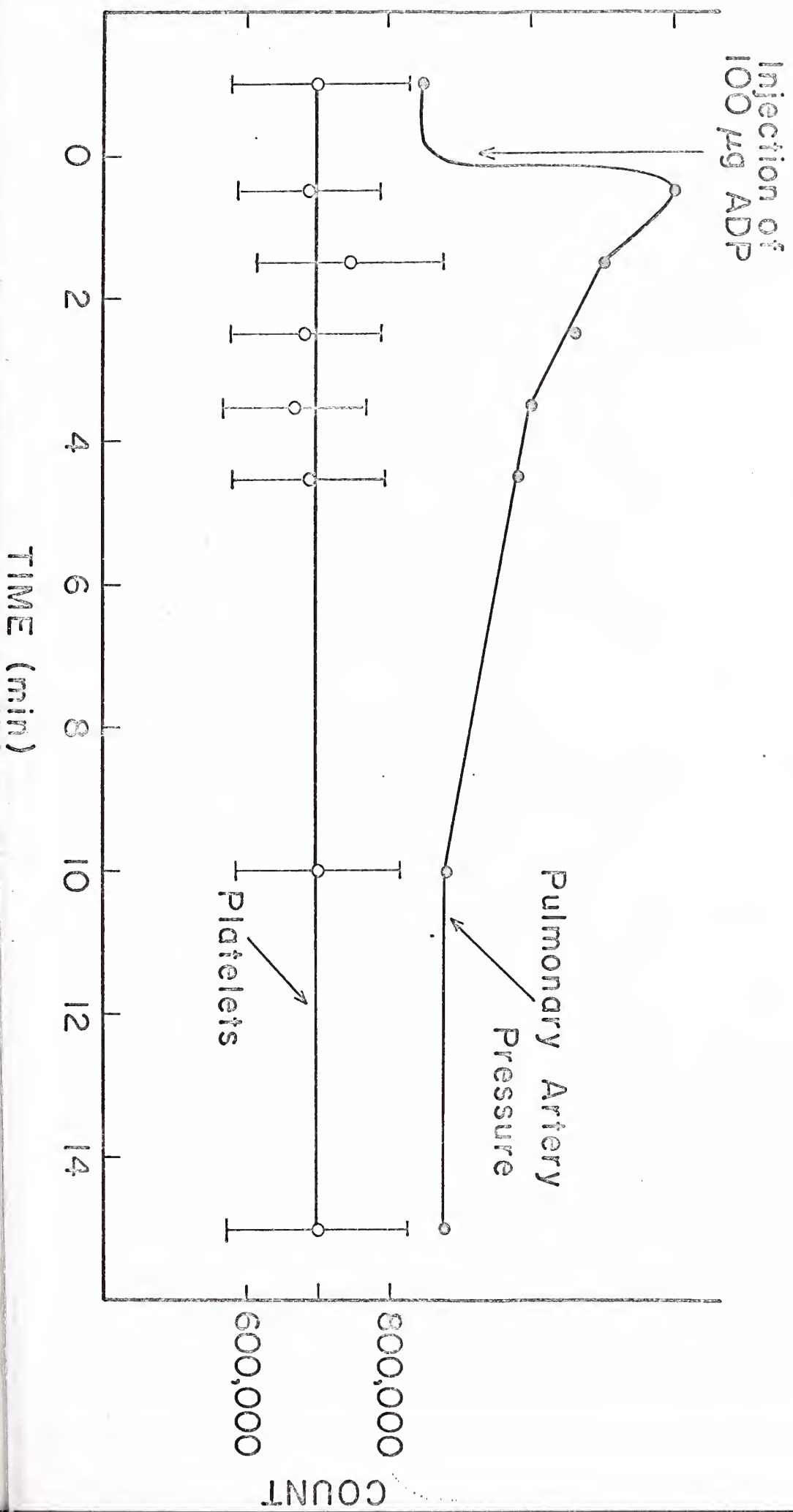
Nucleotide	pH
ATP	6.5
ADP	5.5
AMP	5.6
ITP	5.0
IDP	5.7
IMP	6.6
GTP	5.6
GDP	6.2
GMP	6.5
UTP	5.2
UDP	5.9
UMP	6.5
CTP	5.2
CDP	5.6
CMP	6.6

Figure 8:

Graphic account of Table 6 in appendix showing no statistical significant change in platelet count with 100 ug of ADP induced pulmonary hypertension. Identical results were obtained following the injection of 103 ug of GDP.

Vertical bars on platelet curve (lower line) represents the standard deviation of the individual mean platelet counts.

47 PULMONARY ARTERY PRESSURE BEFORE AND AFTER INJECTION OF 100 μ g OF ADP AND PLATELET COUNT TAKEN AT THE SAME TIME



Platelet counts of calves after infusion of 5 cc of saline into pulmonary artery. Means of platelet counts of eight samples collected during a fifteen minute period.

ANIMAL	NUMBER OF COUNTS	MEAN PLATELET COUNT	STANDARD DEVIATION
1	8	713,100	48,100
2	8	329,700	68,500
3	8	563,100	68,500
4	8	893,800	14,800
5	8	937,500	21,100
6	8	665,300	184,300

Discussion

The results of this study showed that in high and low altitude acclimatized calves pulmonary hypertension of up to sixty-five minutes duration could be induced by high energy nucleotides. The most potent of the nucleotides tested was ADP. Since ADP was known to be a specific aggregator of platelets,³⁵ I considered the possibility that the nucleotide induced hypertension was caused by occlusion of the pulmonary vessels with platelet aggregates. However, no correlation was found between changes in arterial platelet counts and nucleotide induced pulmonary hypertension by ADP. Moreover, GDP which does not aggregate platelets, also caused pulmonary hypertension. In addition, the time course of the nucleotide-induced pulmonary hypertension was not consistent with the hypothesis that the hypertension was secondary to platelet aggregation. Platelet aggregates caused by ADP disperse within a minute in vivo^{69,70} but the pulmonary hypertension persisted for up to sixty-five minutes. The data therefore indicate that the nucleotide-induced pulmonary hypertension is unrelated to platelet aggregation.

Thus, the evidence presented in this paper identifies nucleotides as humoral pressor substances which act specifically on the pulmonary artery pressure. This finding of course does not prove that pulmonary hypertension is caused by these compounds. However, such a possibility cannot be ruled out because ADP is generally present in erythrocytes, leucocytes, and platelets ^{the} in/circulation through the pulmonary vasculature.^{10,71,72}

The regional selectivity of the arterial pressor effect of ADP, was clearly shown as it elevated pulmonary arterial pressure and at the same

time caused a decrease in the systemic blood pressure.

How then does the pressor effect of nucleotides upon pulmonary artery pressure fit into the current hemodynamic concepts of blood pressure regulation in the lungs? In two studies by Blount and Vogel⁹, and Schepers⁷ on high altitude hypertension and pulmonary heart disease ("cor pulmonale"), the authors implied that a humoral transmitter substance may be important in the pathogenesis of increased pulmonary vascular resistance. Their investigations are summarized in the following paragraphs.

Blount and Vogel⁹ found that pulmonary artery pressure increased in normal subjects exposed to a critical level of environmentally induced hypoxia. The critical atmospheric pressure level lay at 7,500 feet or slightly above the altitude at which many million people reside. The physiological status of subjects residing at 10,000 feet, e.g., in Leadville, Colorado, differed from that of persons living below 7,500 feet in that adaptive pulmonary hypertension, represented a common finding in the high altitude subjects. The adaptive pulmonary artery hypertension of the Leadville type was due to increased resistance in the pulmonary circulation, and not to increased pulmonary blood flow. The hypertension could be counteracted by breathing air containing 44 per cent oxygen and by vaso-dilators such as tolazoline.

Blount and Vogel described a number of pathological pulmonary conditions associated with chronic exposure to altitudes above 10,000 feet. These included pulmonary artery hypertension, chronic mountain sickness and the dramatic and dangerous syndrome of high altitude induced acute pulmonary edema. The authors concluded, "A primary increase in alveolar membrane

permeability, induced by some metabolic product or transmitter substance, may be important."⁹

Cor Pulmonale is a clinical condition in which hypertrophy and/or failure of the right ventricle is secondary to increased pulmonary vascular resistance to blood flow. G. W. H. Schepers presented a comprehensive summary of the subject, and distinguished between "primary," "secondary," and miscellaneous" cardiac states caused by pulmonary hypertension. The hypertension was characterized by obstruction in the pulmonary stream bed, and was due either to mechanical interference, e.g. kinking of the artery, massive thrombosis, generalized atheromatosis, or to "functionally induced increases of resistance in the terminal or pre-terminal pulmonary stream bed."⁷

The results of the experiments described in this paper indicate that nucleotides, particularly ADP, represent intermediate metabolites whose pressor effect upon the pulmonary circulation deserves further study.

Summary

- 1) The high energy nucleotides ATP, ADP, GTP, GDP, ITP, IDP, and UDP in doses of 100 ug caused pulmonary hypertension.
- 2) With the exception of UDP, the presso-active compounds were purines.
- 3) None of the pyrimidines other than UDP nor any of the mono-phosphates were presso-active.
- 4) ADP was the most potent presso-active high energy nucleotide.
- 5) Magnitude and duration of response of pulmonary artery pressure was greatest in calves that were acclimatized to simulated altitudes of about 11,000 feet.
- 6) Presso-active nucleotides induce prolonged responses in altitude-acclimatized calves of up to four months of age.
- 7) No correlation was found between changes in arterial platelet counts and nucleotide induced pulmonary hypertension.

APPENDIX

The following represents the statistical method used to evaluate data obtained from the pressor and platelet effects of the high energy nucleotides.

PAIRED T-TESTS WITH MISSING DATA

Purpose

This program produces t-tests with associated probabilities between corresponding pairs of elements of two groups, i.e. variable no. 1 of Group 1 is tested against variable no 1 of Group 2, etc.

Options provided are:

1. t-tests with associated probabilities for uncorrelated data
2. t-tests with associated probabilities for correlated data.
3. missing data is permitted with correlated or uncorrelated data
4. rearrangement of the variables on input is allowed

In uncorrelated t-tests, a test is performed to determine the homogeneity of the variances of the variables, and the appropriate standard error of differences and degrees of freedom are chosen accordingly. The program will handle up to 200 variables in each group.

7B

The following formulas are used in computing:

$$\text{Sum: } \Sigma X_i$$

$$\text{Mean: } \frac{\Sigma X_i}{N_i}$$

$$\text{Variance: } \frac{N_i \Sigma X_i^2 - (\Sigma X_i)^2}{N_i(N_i - 1)} = S_i^2$$

$$\text{Standard deviation: } \sqrt{S_i^2} = SD_i$$

$$\text{Standard error: } \frac{SD_i}{\sqrt{N_i - 1}}$$

$$\text{Coefficient of variation: } \frac{SD_i}{\text{mean}_i}$$

$$\text{T - ratio: } \frac{\text{mean}_i - \text{mean}_j}{\text{standard error of difference}}$$

$$\text{Standard error of difference: } \sqrt{\frac{\text{var}_i + \text{var}_j - 2r_{ij} \sqrt{\text{var}_i \text{var}_j}}{N_{ij}}}$$

Correlated variables -

$$\text{Uncorrelated variables - (Unequal variances) } \sqrt{\frac{\text{var}_i}{N_i} + \frac{\text{var}_j}{N_j}}$$

5

$$\text{Uncorrelated variables - (Equal variances) - } S_p^2 \left(\frac{1}{N_i} + \frac{1}{N_j} \right)$$

$$\text{where } S_p^2 = \frac{(N_i - 1)S_i^2 + (N_j - 1)S_j^2}{N_i + N_j - 2}$$

Degrees of Freedom:

$$\text{for correlated variables - } N_{ij} - 1$$

$$\text{for uncorrelated variables - (unequal variances) - } \frac{S_d^4}{\frac{(S_i^2 / N_i)^2}{N_i + 1} + \frac{(S_j^2 / N_j)^2}{N_j + 1}}$$

$$\text{where } S_d^4 = \frac{S_i^2}{N_i} + \frac{S_j^2}{N_j}$$

$$\text{for uncorrelated variables - (equal variances) - } N_i + N_j - 2$$

Table 5: MEAN PREINJECTION CONTROL PLATELET COUNT VERSUS SUBSEQUENT POSTINJECTION PLATELET COUNT

DRUG	Mean (Pre-Exp.) Platelet Counts	S.D.	Mean Pulmonary Artery Pressure (mmHg)	MEAN VALUES OF PULMONARY ARTERY PRESSURE (0.5 min) Platelet		MEAN P.A.P. (mmHg)
				Count	S.D.	
Saline (6)	751,700	+188,500	25	707,500	+224,200	24
ADP (6)	705,400	+259,000	22	694,600	+200,500	40
GDP (6)	704,600	+209,500	26	735,400	+184,500	41

*P values define numerical relationship between mean post-injection and control platelet counts.

AND PLATELET COUNTS (TIME AFTER INJECTION)

P*	(1.5 min) Platelet Count	S.D.	MEAN		P*	(2.5 min) Platelet Count	S.D.
			P.A.P. (mmHg)				
0.35979	710,800	+216,900	25	0.36750	698,300	+230,900	
0.46851	746,700	+263,000	35	0.39494	678,800	+219,100	
0.39610	717,100	+228,900	31	0.46167	764,200	+300,200	

MEAN			MEAN			
P.A.P. (mmHg)	P*	(3.5 min) Platelet Count	S.D.	P.A.P. (mmHg)	P*	(4.5 min) Platelet Count
25	0.33525	697,500	+254,000	25	0.34188	597,800
33	0.42559	662,900	+211,300	30	0.38093	687,500
30	0.34923	678,700	+227,000	29	0.42089	662,500

S.D.	MEAN			S.D.	MEAN		
	P.A.P. (mmHg)	P*	(10 min) Platelet Count		P.A.P. (mmHg)	P*	(15 min)
<u>+275, 100</u>	25	0.14248	642, 900	<u>+244, 100</u>	24	0.20400	
<u>+215, 200</u>	29	0.44945	692, 100	<u>+233, 400</u>	24	0.46361	
<u>+224, 600</u>	28	0.37203	647, 500	<u>+242, 100</u>	27	0.33577	

Platelet Count	S.D.	MEAN P.A.P. (mmHg)	P*
663,300	<u>+243,800</u>	23	0.24932
708,300	<u>+255,400</u>	24	0.49236
662,100	<u>+251,800</u>	27	0.37857

In an attempt to make the platelet counts more accurate, an effort was made to count platelets by the method described by Bull et al.⁷⁴ This method separates red cells from platelets by sedimentation of EDTA treated whole blood in small bore plastic tubes. During sedimentation, the red cells, probably because of their surface charge, trap some plasma which is free of platelets. This leads to an excess of platelets in the supernatant plasma which is then later corrected for by knowing the hematocrit value. Once the plasma layer has formed, a 3 ul capillary pipet is filled from the plasma layer and diluted. This solution is counted with a 70 micra-aperture attached to a model A Coulter counter. This method is said to have a coefficient of variation of only 3.8 per cent. Unfortunately, the above method could not be adopted to this experiment since it soon became evident that the sedimentation rate of calf blood averaged less than 0.5 mm per hour and the plasma layer formed so slowly that by the time a sufficient amount accumulated for counting purposes, the platelets had undergone degeneration and the machine counts showed fluctuations on a single sample of greater than 50 per cent.

Finally, platelets were counted by the method of Ingram⁷⁵ in which the platelet rich plasma is separated from the red and white blood cells by placing the blood sample to be counted above a layer of Dow Corning silicone with a specific gravity of 1.0680 in a microhematocrit tube. When this microhematocrit tube is centrifuged, the red blood cells due to their greater specific gravity

move through the silicone layer, leaving behind a platelet rich plasma which following dilution is counted with a Coulter counter in a manner similar to that described above. Again, this method could not be utilized since it appears that the calf red and white blood cells have a density which is not sufficient to precipitate them through the silicone layer which has been found to be effective for separating dog and rat blood. Thus the counts with this method also fluctuated widely due to the red and white cell fragments which remained in the diluted sample used to count the platelets. However on discussion with Dr. Ingram, it appears that this method should be promising once the specific gravity of the silicone separating solution is so adjusted to allow the calf red and white cells to sediment through it during centrifugation, while the platelets are left in the supernate.

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