The effect of cyclic AMP on the activity of the purified protein kinase as a function of incubation time is shown in Fig. 1. Cyclic AMP increased the initial rate of phosphorylation of histone. The rate in the presence of cyclic AMP was linear for about 5 minutes. The activity of the protein kinase was proportional to enzyme up to 45 μg, the highest amount tested.

The effect of the concentration of cyclic AMP on the activity of the protein kinase is shown in Fig. 2. Near maximal stimulation was obtained with about $5 \times 10^{-7}M$ cyclic AMP. It is clear that Mg^{2+} ions are required for the kinase activity dependent on cyclic AMP. (The presence in the purified preparation of protein kinase activity not dependent on cyclic AMP is also indicated by the data in Fig. 2.) In other experiments, designed to determine the apparent K_m, half-maximum stimulation of histone phosphorylation was achieved at a concentration of cyclic AMP of $8 \times 10^{-8}M$. This value is similar to the values for the cyclic AMP–dependent protein kinase from muscle (*10*) as well as that from liver (*11*). Adenosine 5′-monophosphate, in concentrations from 5×10^{-8} to $5 \times 10^{-6}M$, could not replace cyclic AMP for the activation of the protein kinase of rabbit brain.

A comparison of the ability of various proteins to serve as substrate for the purified protein kinase preparation is shown in Table 1. Protamine was able to serve as a phosphate acceptor, but was much less effective than histone, and phosvitin was still less effective. Langan (*11*) found protamine to have little or no ability to serve as phosphate acceptor for the cyclic AMP-dependent histone kinase from liver. The protein kinase from skeletal muscle, prepared according to the procedure of Walsh, Perkins, and Krebs (*10*), also phosphorylates histone much more readily than protamine (*13*). Thus, the cyclic AMP-dependent protein kinases found in liver, muscle, and brain all use histone preferentially to protamine as substrate.

The demonstration of a cyclic AMP–dependent protein kinase in brain tissue would appear to be the first evidence of a biochemical or physiological effect of cyclic AMP in nervous tissue and, taken together with data (*5–7*) indicating changes in brain cyclic AMP levels in response to various neurohumors or electrical stimulation, provides rather strong evidence for a role of cyclic AMP in brain function. The nature of the interaction between cyclic AMP and protein kinase must await further purification of the enzyme. However, the recent demonstration that cyclic AMP is a high-energy compound, with a free energy of hydrolysis substantially greater than that of ATP, and that it is capable of adenylylating pyrophosphate, makes it feasible to consider the possibility that cyclic AMP interacts with the protein kinase by adenylylating it (*14*). Another interesting problem, raised by the finding of a protein kinase in brain tissue, is whether this protein kinase is the only "receptor" with which cyclic AMP interacts in the brain. Finally, further investigation is required to determine the nature of the naturally occurring substrate for protein kinase in brain tissue.

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Aspirin: Its Effect on Platelet Glycolysis and Release of Adenosine Diphosphate

Abstract: *Incubation of human platelets with aspirin inhibited glycolysis and produced a fall in the concentration of adenosine triphosphate. When platelets were exposed to collagen there was an increase in glycolysis and release of adenosine diphosphate. Prior incubation of the platelets with aspirin for 5 minutes did not totally suppress the increase in glycolysis after exposure to collagen but completely inhibited the collagen-induced reaction of the release of adenosine diphosphate. It is suggested that aspirin acts on human platelets by inhibiting both release of adenosine diphosphate and the transport of glucose across the platelet membrane.*

The fundamental contribution of platelets to hemostasis and thrombosis is well recognized. When a vessel is injured, platelets adhere to the exposed subendothelial collagen, release adenosine diphosphate (ADP), and then aggregate (*1*). The importance of this reaction has recently been emphasized by the demonstration that aspirin and other drugs that impair the release, by collagen, of platelet ADP in vitro inhibit thrombus formation and hemostatic plug formation in vivo (*2, 3*).

The effect of salicylates on platelet metabolism is unknown; however, salicylates have been reported to interfere with carbohydrate metabolism in several other tissues by uncoupling oxidative phosphorylation (*4*), inhibiting erythrocyte glycolysis (*5*), and increasing liver and muscle glycogenolysis (*6*).

The possibility exists that aspirin also interferes with platelet carbohydrate metabolism and that through this effect inhibits release of ADP. Alternatively, aspirin may specifically inhibit the release of platelet ADP without interfering with other metabolic changes caused by the interaction of platelets with collagen.

These questions were examined by first investigating the influence of aspirin on platelet carbohydrate metabolism and then by comparing its effect on release of platelet ADP with its

Table 1. Effect of aspirin and collagen on platelet metabolism. Results are expressed as micromoles per 10^{11} platelets, and represent the means ± standard deviation of five experiments. Glucose concentration was 100 mg per 10^{11} platelets. The addition of 2 m*M* sodium cyanide in the oxygen experiments rapidly reduced oxygen uptake to zero, confirming that the observed changes in oxygen partial pressure represented true mitochondrial activity.

Metabolism measured (per hour)	Control		Aspirin treatment	
	Collagen absent	Collagen added	Collagen absent	Collagen added
Lactate production	169 ± 24	201 ± 22	88 ± 30	106 ± 43
Percentage of control value	100	119	52	63
Glucose uptake	99 ± 19	133 ± 17	40 ± 12	71 ± 13
Percentage of control value	100	134	40	72
Glycogen utilization	17.5 ± 4.2	18.3 ± 3.9	25.4 ± 5.6	25.9 ± 5.0
Percentage of control value	100	105	145	148
Oxygen uptake	26 ± 4	25 ± 8	34 ± 3	
Percentage of control value	100	97	129	
ATP level after incubation for 1 hour				
	7.1 ± 0.6		4.5 ± 0.4	
Percentage of control value before incubation				
	100		63	

effect on the increase in platelet glycolysis produced by collagen.

Platelet aggregation and release of ADP were measured in citrated platelet-rich plasma (PRP) as previously described (*7*). Suspensions of washed platelets (1 to 2 × 10^6 per cubic millimeter) were prepared from human blood according to the method described by Doery, Hirsh, and de Gruchy (*8*). Glucose was added at a ratio of 100 mg per 10^{11} platelets. Stock aspirin solutions (110 mg/ml) were prepared daily in the same modified Ringer solution used for the platelet suspensions and brought to *p*H 7.4 with anhydrous sodium carbonate before use. Lyophilized collagen (Sigma, U.S.A.) was added to cold Tyrode's solution (10 mg/ml) and subjected to ultrasonic disintegration at 4°C for repeated short intervals over 1 hour. The slightly opaque supernatant was decanted and used for the collagen experiments. Carbohydrate metabolism of platelets was investigated by measuring glucose uptake, glycogen consumption, lactate production, oxygen consumption, and ATP levels with the appropriate additions of aspirin or collagen. Portions of platelet suspension were incubated at 37°C in unstoppered centrifuge tubes. At the completion of the incubation time the tubes were placed in melting ice and agitated for 1 minute. A platelet button for glycogen analysis was then prepared by centrifugation at 2500*g* for 7 minutes at 0°C. The supernatant was removed and a portion added to an equal volume of cold 7 percent perchloric acid and frozen overnight at − 20°C. This was thawed to 4°C the following day and centrifuged to remove precipitated aspirin; lactate determinations were carried out on the supernatant by the use of an enzymatic method (*7*). Glucose determinations were performed on the same samples, after neutralization with anhydrous sodium bicarbonate, by the hexokinase method of Pfleiderer (*9*). For glycogen determinations the residual platelet button was treated as follows: 2 ml of 45 percent potassium hydroxide was added and digestion carried out by heating for 20 minutes in a boiling water bath; 4 ml of absolute ethanol was added and the tubes were heated just to the point of boiling and then cooled to − 20°C for 30 minutes. The precipitated glycogen was then recovered by centrifugation at 2200*g* for 30 minutes at 0°C and washed twice with 3 ml of absolute ethanol. This was then hydrolyzed by heating for 2 hours with 2*N* H_2SO_4. The liberated glucose was assayed as above (*9*). For determination of the concentration of platelet ATP the tubes were removed from the water bath after incubation at 37°C, plunged into a mixture of ice and salt, and an equal volume of cold 7 percent perchloric acid was added rapidly. This was followed by ultrasonic disintegration for 20 seconds; the samples were then stored frozen overnight at − 20°C. After thawing and centrifugation, the supernatant was adjusted to *p*H 7.5 and adenosine triphosphate (ATP) was assayed by the same enzymatic method mentioned above (*7*). The effect of collagen on the distribution of particle-bound and cytoplasmic hexokinase was investigated as described previously (*10*). Ultrasonic disintegration was used to disrupt the cells. Oxygen consumption was measured in platelet suspensions incubated at 37°C by monitoring changes in oxygen partial pressure over a period of approximately 20 minutes with a Clark-type platinum electrode. In all aspirin experiments the platelet suspensions were preincubated with the drug for 5 minutes before sampling or addition of collagen. The concentration of aspirin chosen was one that totally inhibited platelet aggregation and ADP release in citrated platelet-rich plasma by a very active collagen suspension (Fig. 1A). This concentration, approximately 56 m*M*, is about 40 times higher than the upper therapeutic concentrations in blood. However, doses as low as 1 m*M*, which only partially inhibited aggregation by less active collagen (for example, prepared by dilution as shown in Fig. 1B) produced effects on lactate production and glucose uptake similar to the higher concentrations. The biochemical changes produced by aspirin, collagen, and aspirin plus collagen are shown in Table 1. Incubation of the platelet suspensions with aspirin significantly affected glycolysis. Thus lactate production was decreased by 48

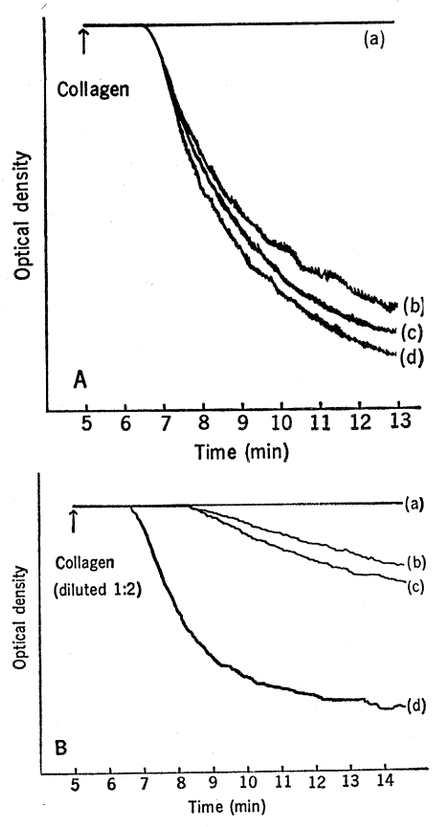

Fig. 1. The effects of the concentration of aspirin on aggregation of citrated PRP with undiluted collagen (A) and collagen diluted 1:2 (B). Aspirin was preincubated with the platelet suspension for 5 minutes at 37°C before the addition of collagen. Concentration of aspirin: curve a, 55.5 m*M*; curve b, 11.1 m*M*; curve c, 2.2 m*M*; and curve d, 0.

percent ($P < .01$) and glucose uptake by 60 percent ($P < .01$) of basal levels. On the other hand, glycogen consumption was increased by 45 percent ($P < .01$) and oxygen consumption by 29 percent ($P < .1$) above basal activity. These changes were associated with a 37 percent fall in platelet ATP over 1 hour (Table 1). The decreased glycolysis and the associated increase in glycogenolysis suggest that aspirin inhibits glycolysis at a point between extracellular glucose and glucose-6-phosphate. Preliminary studies have failed to reveal an effect of aspirin on platelet hexokinase assayed in vitro, which suggests that aspirin suppresses glucose transport. The increase in oxygen uptake may be a consequence of the impaired glycolysis and fall in ATP levels or may be due to an uncoupling of oxidative phosphorylation (*4*). The mechanism by which aspirin produces its effects on platelets is uncertain. Aspirin has been shown to acetylate numerous human proteins (*11, 12*) and it is possible that it inhibits ADP release and glucose transport by acetylation of membrane proteins.

Collagen produced a 19 percent increase in lactate production ($P < .05$) and a 34 percent increase in glucose uptake ($P < .05$), but no effect on glycogen utilization or oxygen uptake (Table 1). In addition, collagen produced release of ADP and redistribution of particle-bound hexokinase into the cytoplasm (*10*). The effect on both lactate production and release of ADP was markedly reduced when the collagen preparation was heated to 55°C or incubated with collagenase (0.1 mg/ml; 37°C) to the point where it just failed to produce visible aggregation. In a series of four experiments, heat denaturation reduced the increase in lactate production from 35 to 6 percent and release of ADP from 1.7 to 0.1 μmole per 10^{11} platelets. Collagenase treatment reduced the increase in lactate production to 4 percent and the release of ADP to 0.1 μmole per 10^{11} platelets. This suggests that the effects on both platelet aggregation and glycolysis are caused by a common property of the collagen fiber.

Aspirin completely inhibited ADP release but did not prevent the effects of collagen on glycolysis and particle-bound hexokinase. This suggests that aspirin does not suppress the overall interaction of platelets with collagen but that it interferes with certain steps in the platelet collagen reaction. Collagen is thought to produce a generalized increase in platelet membrane permeability (*13*) and it may be that it overcomes the inhibitory effect of aspirin on glucose transport by such a mechanism. On the other hand, an increase in membrane permeability would not necessarily cause release of ADP because it has been shown that only the ADP present in granules and not that in cytoplasm is released by collagen (*14*).

It thus appears that aspirin acts on human platelets in at least two ways: by inhibiting release of ADP and inhibiting glucose transport. The inhibition of ADP release is unlikely to be a direct result of the decreased glycolysis and energy production.

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Tadpole Antibodies against Frog Hemoglobin and Their Effect on Development

Abstract. Rana catesbeiana *tadpoles immunized with the main hemoglobin component of the adult frog of the same species produced precipitating and agglutinating antibodies against the immunogen. After natural metamorphosis, the immunized froglets have as their only major hemoglobin a protein immunologically and electrophoretically different from the major hemoglobin of control froglets.*

During the metamorphosis of *Rana catesbeiana* tadpoles the larval hemoglobin is replaced by adult hemoglobin, a protein with a different peptide chain constitution (*1, 2*); rabbit antibodies against either hemoglobin do not cross-react with the other (*3, 4*). It has now been demonstrated that tadpoles form precipitating antibodies when their own adult hemoglobin is used as the immunogen. These animals are particularly likely to show this phenomenon because the larval stages are immunologically competent (*5*) and because the major frog hemoglobin was not detectable immunologically in tadpoles before metamorphosis (*4*).

Larval and adult *R. catesbeiana* were obtained from the Connecticut Valley Biological Supply Co. Tadpoles were immunized with the dimer of the major frog hemoglobin, which had been fractionated by gel filtration on Sephadex G-100 (*2*). The dimer (5 μg) was emulsified in complete Freund's adjuvant and injected subcutaneously into the backs of tadpoles before metamorphosis. One month later a booster injection of the same amount was given intraperitoneally without adjuvant. Two months after the first injection, serum was obtained by decapitation of three immunized tadpoles and pooled and concentrated to one-fifth of the original volume by lyophilization. The concentrated antiserum tested against the immunogen by double diffusion in agar gave a single precipitin line. Since the animals do not come from an inbred strain, we tried to obtain evidence against the presence in the immunogen of an unusual hemoglobin variant. The concentrated tadpole antiserum was tested against hemoglobins from frogs supplied by three different companies. A single precipitin line developed in all cases (Fig. 1A), whereas with concen-