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The Effect of Epinephrine on the *in Vivo* Concentration of Erythrocyte Glycolytic Intermediates (39562)

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The studies reported herein were initiated when we observed an increase in the concentration of erythrocyte adenosine triphosphate (ATP) in a rhesus monkey which had escaped from its cage and was recaptured after a 10- to 15-min chase. This observation led to experiments, which will be presented in this paper, dealing with the effect of epinephrine injections on rhesus erythrocyte glycolytic intermediate concentrations. A pharmacological agent which increases red cell ATP might accomplish this by stimulating erythrocyte glycolysis. Stimulation of glycolysis can potentially increase erythrocyte ATP and 2,3-diphosphoglycerate (DPG) concentrations which in turn may enhance erythrocyte function. ATP is generated by the glycolytic process and is important in maintaining normal shape (1, 2) and deformability (3) of the red cell. The red cell also uses ATP to maintain physiological intracellular concentrations of Na^+, K^+ (4), and Ca^{2+}. DPG, another important molecule generated by glycolysis, increases the release of oxygen from hemoglobin to the tissues by decreasing the oxygen affinity of hemoglobin (5, 6).

Materials and methods. In initial experiments, male rhesus monkeys were anesthetized by im injection of phencyclidine hydrochloride (Sernylan, Parke-Davis Co.). In later experiments, the monkeys were anesthetized by a combination of ketamine hydrochloride (Ketalar, Parke-Davis Co.) given im at a dose of about 20 mg/kg body wt and pentobarbital given slowly by the iv route at a dose of 5–10 mg/kg body wt. Ketalar alone was found to be just as effective as the combination of anesthetics and thus was used alone in most experiments. The type of anesthetic used is designated by footnotes in the tables.

Two milliliters of epinephrine solution (1 mg/ml) was given ip to anesthetized monkeys, followed 15 min later by another 1.5 ml. All of the monkeys studied weighed about 5.0 kg, thus the 3.5 mg of epinephrine represented about 0.7 mg/kg. Controls were injected with an equivalent volume of saline. About 12 ml of blood were drawn from anesthetized monkeys 30 min after the first epinephrine or saline injection. The blood was drawn into heparinized syringes via puncture of the femoral vein and a portion precipitated immediately for the determination of ATP, DPG, and glycolytic intermediates. In some of the early experiments, glycolytic intermediates were not assayed.

The blood precipitation procedures have been described previously and employ trichloroacetic acid for the precipitation of ATP and DPG, and perchloric acid for the precipitation of the remaining glycolytic intermediates. The assay of ATP follows the method of Brewer and Powell (7) and the DPG assay is that of Keitt (8). The method of preparation and assay of extracts for the remaining glycolytic intermediates is a modification of Oelshlegel *et al.* (9) of that described by Minakami *et al.* (10). Leukocytes and platelets are not removed before the blood is precipitated because the contribution of these cells to the concentrations of glycolytic intermediates in blood has been found to be negligible (10).

In some of the early epinephrine injection experiments, the blood used to determine control levels of ATP, DPG, and glycolytic intermediates was drawn from the same monkeys 9 days before or after the blood used to determine epinephrine-treated levels of these variables. This scheme was initially employed because we suspected that removal of the blood per se for determination of control values might influence the

level of variables of interest in blood drawn minutes later for postepinephrine values. This precaution was found to be unnecessary, so in subsequent experiments the control blood was drawn from anesthetized monkeys 15 min before the first epinephrine injection, and then the usual protocol was followed.

Epinephrine-induced hyperventilation might stimulate glycolysis via alkalosis and thus would complicate the interpretation of the epinephrine effect. Therefore, in two experiments, anesthetized monkeys were intubated endotracheally in order to maintain a constant blood pH during the subsequent eqinephrine treatment. In one of these experiments, the animal breathed air supplemented with 3% CO_2 for this purpose, and in the other, a small animal respirator was used to control respiration during the course of the experiment.

Results. As mentioned above, we observed that red blood cells from a monkey which escaped and was chased virgorously for 10–15 min had a much higher ATP concentration than was found in the same monkey's red cells when blood was taken under normal resting conditions. In three pilot experiments, we found deliberate vigorous (but not slow) chasing of monkeys also increased their red cell ATP concentration (data not shown).

We did not extend these qualitative observations by obtaining enough data for statistical testing because the chasing experiments were difficult for both animals and investigators. Instead, we turned to experiments designed to determine if epinephrine injected ip into monkeys would produce a similar change in red cell ATP concentration. Table I presents the results of 11 experiments using six 5-kg male monkeys injected with 3.5 mg of epinephrine as described in the Methods section. From Table I it is apparent that epinephrine injections produced a consistent and statistically significant increase in red cell ATP concentration. The average values of control and postepinephrine blood samples were 4.77 and 5.70 μmole of ATP per g of hemoglobin, respectively. The average increase in ATP concentration was 19.5% and the difference between control and epinephrine ATP mean concentrations is significant at the 1% probability level ($P = 0.0144$). The DPG response varied in these experiments and no significant change could be discerned. Prevention of hyperventilation by use of a respirator, or respiration with 3% CO_2 to prevent alkalosis, did not block the increase in ATP induced by epinephrine (Table I).

The effect of epinephrine injection on the levels of glycolytic intermediates in rhesus monkey erythrocytes has been studied in several experiments in order to identify the enzymatic step or steps responding to epinephrine. These experiments are presented in Table II. Epinephrine injection consistently increased the concentration of early red cell glycolytic intermediates, glucose 6-phosphate (G6P), fructose 6-phosphate (F6P), dihydroxyacetonephosphate (DHAP), as well as increasing the ATP concentration. The mean fructose 1,6-diphosphate (FDP) for the six experiments is also increased. Experiment 4 is a time-course experiment for the effect of epinephrine on the glycolytic intermediates. The two injections of epinephrine were followed by a sequence of blood drawings at the usual 30 min after the first injection; in addition, blood was also drawn at 60 and 120 min after the first injection. It is apparent that G6P and ATP levels continued to increase throughout the experiment. In experiment 5, the monkey was intubated endotracheally and ventilated with air supplemented with 3% CO_2 in order to maintain constant blood gas and blood pH during the epinephrine treatment. From the data in Table II, it is evident that G6P, F6P, FDP, DHAP, and ATP are increased subsequent to the epinephrine injections under conditions where the blood CO_2 level is being controlled. In experiment 6, hyperventilation (and alkalosis) was prevented by controlling ventilation at 1.4 liters (40 breaths) per min throughout the experiment by a small animal respirator. Again G6P, F6P, FDP, DHAP, and ATP were elevated after the epinephrine injections. The whole blood pH values presented in the treatment column of Table II (experiments 5 and 6) indicate that respiratory alkalosis is not required for the elevation of G6P, F6P, FDP, DHAP, or

TABLE I. THE EFFECT OF EPINEPHRINE INJECTIONS ON THE CONCENTRATION OF ATP AND DPG IN MALE RHESUS MONKEY RED CELLS.

	ATP (μmole per g of hemoglobin)		DPG (μmole per g of hemoglobin)		Hemoglobin (g%)	
Monkey No.	Control	Epinephrine	Control	Epinephrine	Control	Epinephrine
266[a]	3.75	5.33	–	–	12.64	12.50
269[a]	4.33	5.17	16.21	18.08	12.99	14.11
283[a]	5.93	6.14	16.14	16.81	11.37	12.36
284[a]	4.75	5.31	19.83	19.85	11.94	11.73
286[a]	4.92	5.31	19.66	19.51	10.81	14.11
286[a]	4.67	8.45	20.16	19.20	11.02	13.90
286[b]	4.68	5.68	19.28	24.97	11.72	10.25
287[a]	4.70	4.87	19.51	17.42	12.64	12.15
287[b]	4.22	4.48	14.00	15.94	14.01	14.55
284[b,c]	5.98	6.29	24.04	24.11	8.64	11.02
286[b,d]	4.54	5.71	16.60	20.56	11.23	12.78
Mean	4.77	5.70	18.54	19.64		
Mean difference ± SE[e]	0.93 ± 0.31		1.10 ± 0.74			
Paired Student's t statistic	2.956		1.49			
Significance of difference	$P = 0.0144$		$P = 0.169$			

[a] Control and epinephrine treatment on different days.
[b] Control blood drawn 15 min before first epinephrine injection.
[c] Respirator experiment, control blood pH was 7.470; postepinephrine pH was 7.338.
[d] Three-percent CO_2 experiment, control blood pH was 7.363; postepinephrine pH 7.358.
[e] SE = Standard error.

Note. Since some of the monkeys were used in more than one experiment, the mean control values was compared to the mean treatment value for each monkey. The average inrease in ATP concentration was 17% ($P = 0.026$).

ATP. The phosphorylated intermediates in the lower part of the glycolytic pathway (3-phosphoglycerate and phosphoenolpyruvate) do not change in any consistent way after epinephrine injection. In all of these experiments, except experiment 3, the adenosine diphosphate (ADP) has decreased subsequent to epinephrine injection. The total adenine nucleotide pool has increased except in experiment 6 where the pool decreased by 2.4% due to the relatively large decrease in ADP. The mean increase in the adenine nucleotide pool is 10.9%. This increase derives from the increased ATP.

Blood pyruvate and lactate are a result of metabolism in blood cells and various other tissues in the body, since these metabolites can cross cell membranes. Modest increases in blood pyruvate and lactate concentration were generally found subsequent to the epinephrine except in experiment 5 of Table II (the 3% CO_2 experiment) where both pyruvate and lactate decreased in concentration. The lactate/pyruvate ratios have not been tabulated, but this ratio consistently decreased following the epinephrine treatment. In experiment 3, the lactate/pyruvate ratio after treatment was only 12% of its control value.

In two experiments the epinephrine-induced increases in the concentrations of red cell G6P, F6P, and ATP were not delayed or reduced by the administration of 300 mg of aspirin plus 7.5 mg of indomethacin to a 6.5-kg monkey at 4 hr and again at 15 min before the initial blood was drawn for determination of control levels of glycolytic intermediates (data not shown). In two additional experiments, using one-tenth as much epinephrine (0.35 ml of 1 mg/ml of epinephrine injected into each of two monkeys weighing 4.8 and 6.5 kg), no changes occurred in the glycolytic intermediates or ATP (data not shown). Subcutaneous injection of monkeys with prostaglandin E_2 (in 95% ethanol) at doses of 3 mg/5.25-kg monkey and 5 mg/6.5-kg monkey did not cause a consistent change in G6P, F6P, or ATP (data not shown).

Discussion. In 11 different *in vivo* experiments using six 5-kg rhesus monkeys in-

TABLE II. THE EFFECT OF EPINEPHRINE ON MONKEY ERYTHROCYTE GLYCOLYTIC INTERMEDIATES.

Expt. No.	Monkey No.	Treatment	Concentration in μmol/g of hemoglobin[a,b]											
			G6P	F6P	FDP	DHAP	DPG	3PG	PEP	PYR	LACT	AMP	ADP	ATP
1[c]	286 (4.8 kg)	Control[f]	0.111	0.036	0.023	0.064	19.66	0.86	0.25	0.579	23.7	0.058	0.515	4.92
		Epinephrine	0.206	0.060	0.163	0.323	19.51	0.150	0.043	1.47	33.6	0.052	0.473	5.31
2[c]	284 (5.0 kg)	Control[g]	0.115	0.033	0.076	0.178	19.83	0.387	0.139	–	25.8	0.062	0.499	4.75
		Epinephrine	0.190	0.185	0.076	0.195	19.85	0.127	0.032	–	65.1	0.040	0.488	5.31
3[d]	286 (4.8 kg)	Control[h]	0.100	0.045	0.055	0.065	19.28	0.252	0.061	0.031	5.58	0.052	0.482	4.68
		Epinephrine	0.245	0.128	0.037	0.152	24.97	0.287	0.068	1.55	34.0	0.060	0.514	5.68
4[d]	286 (5.0 kg)	Control (av of Expts. 1 and 3)	0.105	–	0.039	0.064	19.47	–	–	–	14.6	–	–	4.80
		Epinephrine 30 min	0.146	–	0.020	0.081	22.92	–	–	–	35.6	–	–	5.07
		Epinephrine 60 min	0.274	–	0.047	0.180	25.67	–	–	–	38.0	–	–	5.87
		Epinephrine 120 min	0.285	–	0.028	0.048	23.55	–	–	–	31.9	–	–	6.49
5[d,e]	286 (4.8 kg)	Control[h] (pH = 7.363)	0.156	0.038	0.020	0.047	16.60	–	0.032	1.54	42.6	0.078	0.613	4.54
		Epinephrine + 3% CO_2 (pH = 7.356)	0.226	0.066	0.028	0.114	20.56	0.171	0.054	1.11	28.7	0.076	0.526	5.71
6[d,e]	284 (5.0 kg)	Control[h] (pH = 7.470)	0.100	Trace	0.007	0.017	24.04	0.315	0.024	–	44.8	0.094	0.789	5.98
		Epinephrine + respirator (pH = 7.338)	0.266	0.097	0.013	0.045	24.11	0.302	0.038	–	67.1	0.055	0.356	6.29
	Means	Control	0.116	0.038	0.036	0.074	19.88	0.453	0.101	0.717	28.50	0.068	0.580	4.97
		Epinephrine (30 min)	0.213	0.107	0.083	0.151	21.98	0.216	0.047	1.377	44.02	0.056	0.471	5.56

[a] Abbreviations: G6P, glucose 6-phosphate; F6P, fructose 6-phosphate; FDP, fructose 1,6-diphosphate; DHAP, dihydroxyacetone phosphate; DPG, 2,3-diphosphoglycerate; 3PG, 3-phosphoglycerate; PEP, phosphoenolpyruvate; PYR, pyruvate; LACT, lactate; AMP, adenosine monophosphate; ADP, adenosine diphosphate, ATP, adenosine triphosphate.

[b] All experiments except no. 4 have provided ATP and DPG data for Table I.

[c] Anesthetized with Sernylan.

[d] Anesthetized with Ketalar.

[e] Anesthetized with pentobarbital.

[f] Control blood drawn 9 days after epinephrine treatment and bleeding.

[g] Control blood drawn 9 days before epinephrine treatment and bleeding.

[h] Control (baseline) blood drawn 15 min before first epinephrine injection.

jected with 3.5 mg of epinephrine, we have found a consistent and statistically significant ($P = 0.0144$) increase in monkey red cell ATP concentration. The average increase in red cell ATP was 19.5%. The increase which we have observed in the total pool of adenine nucleotides is dominated by the ATP increase. One possible explanation of this increase could be an increase in red cell glycolytic activity since ATP is a product of glycolysis. We think these increases are due to increased glycolysis and probably some increase in adenosine monophosphate synthesis as well. *In vitro* measurement of red cell glucose consumption or lactate production can be used to establish increased or decreased glycolytic rate. *In vivo*, however, it is not possible to measure glycolytic rate in any direct way, and the rate of glycolysis after the blood is drawn may not reflect the *in vivo* status.

However, a change of *in vivo* enzymatic activity may be detected by a change in the concentration pattern of glycolytic intermediates. The mean concentration of each of the early glycolytic intermediates after epinephrine injection expressed as a percentage of its mean control concentration is 184, 282, 231, and 204 for G6P, F6P, FDP, and DHAP, respectively. Thus, on the average, each of these four intermediates has experienced about the same relative increase in association with the epinephrine injection. These twofold increases in the *in vivo* concentrations of rhesus red cell G6P, F6P, FDP, and DHAP, which we have observed subsequent to epinephrine injection, are compatible with an *in vivo* stimulation of red cell hexokinase activity by the epinephrine or some substance derived from epinephrine or its physiological action. However, alternative interpretations need to be evaluated. Phosphofructokinase (PFK) is an important regulatory enzyme in glycolysis (11), but we do not believe this enzyme is responding to the epinephrine injection in our experiments because, as stated above, its substrate (F6P) and its product (FDP) have both increased by about the same relative amounts. Stimulation of PFK would be expected to increase FDP and DHAP and decrease G6P and F6P relative to their respective control concentrations (or at least increase the former two more than the latter two), but this was not found in our experiments. Also free ATP is an inhibitor of PFK (11) but if the increased ATP concentration subsequent to epinephrine injection was inhibiting PFK significantly, we should see decreased FDP and DHAP relative to G6P and F6P. Thus, inhibition of this regulatory enzyme due to increased red cell ATP concentration is apparently not occurring under these particular conditions. Therefore, our interpretation is that these changes in glycolytic intermediates and adenine nucleotides, subsequent to epinephrine injection, are most logically explained as the result of some kind of stimulatory effect on red cell hexokinase.

The concentrations of 3-phosphoglycerate and phosphoenolpyruvate, which are in the lower part of the glycolytic pathway, did not change in a consistent way. Since phosphate is required at the glyceraldehyde 3-phosphate dehydrogenase (GAPD) enzymatic reaction of glycolysis, it is conceivable that a variable decrease in blood phosphate concentration could be an explanation for the variable decrease in red cell 3-phosphoglycerate and phosphoenolpyruvate after epinephrine injection. We have not assayed for blood phosphate concentrations in any of our experiments and cannot eliminate the possibility that epinephrine might have induced certain body tissues to utilize phosphate more vigorously.

The GAPD reaction consumes nicotinamide adenine dinucleotide (NAD^+) and produces the reduced form of this cofactor (NADH). A decrease in the NAD^+/NADH ratio could presumably exert inhibition on the GAPD step (12). The NAD^+/NADH ratio can be decreased by an *increase* in the lactate/pyruvate ratio due to the near equilibrium condition at the lactate dehydrogenase enzymatic step (12). Blood lactate and pyruvate may come from various tissues as stated earlier. However, this source of GAPD inhibition cannot be logically invoked to explain our glycolytic intermediate pattern changes associated with epinephrine injection because the lactate/pyruvate ratio changes in the wrong direction (it decreases, which would tend to increase the NAD^+/NADH ratio). If the lactate/pyruvate ratio

were to have any significant bearing on our results, it should be to help rather than hinder GAPD *in vivo* activity. Since the glycolytic intermediate pattern changes we have found do not argue for GAPD stimulation, we conclude that changes in lactate, pyruvate, and their ratio probably have no significant bearing on our results.

The epinephrine treatment caused an apparent hyperventilation and, thus possibly, some decrease in blood CO_2 level and increase in blood pH. An increase in pH might be the cause of a glycolytic response. However, when the possible blood pH increase was prevented, either by ventilation with air and 3% CO_2, or by a respirator, epinephrine treatment led to essentially the same increases in concentration of red cell G6P, F6P, DHAP, and ATP as usually seen. Thus, hyperventilation does not appear to have been the cause of the increase in these metabolites.

There are at least two different theoretical mechanisms by which epinephrine might alter rhesus red cell glycolysis. Harrison and Gray (13, 14) have reported that various catecholamines at concentrations in the 10^{-5} to 10^{-3} *M* range stimulate yeast hexokinase and hexokinase from brain homogenates *in vitro* when Mg^{2+} concentration and pH were suboptimal and when the concentration of glucose exceeded the half-saturation level. The 3.5-mg dose that we injected into the monkeys would produce a calculated peak theoretical epinephrine concentration of 8.5×10^{-5} *M* in the plasma if the entire dose went immediately into the blood stream. If the epinephrine diffused into their red cells, the resulting monkey whole blood concentration would be 6.3×10^{-5} *M*. The analogous theoretical concentration associated with the 0.35-mg epinephrine dose, which did not change glycolytic intermediates or adenine nucleotides, would be one-tenth as great. Human erythrocytes have been reported to take up epinephrine (15) and various amines (16). Roston (15) observed rapid penetration of small amounts of epinephrine and norepinephrine into erythrocytes. If this penetration also occurs with rhesus red cells, the *in vivo* concentration of epinephrine inside the cell which is obtained may be more than the minimum required for hexokinase stimulation and it might explain the altered levels of ATP and glycolytic intermediates in the epinephrine-injected monkeys.

An additional mechanism for glycolytic stimulation is suggested by the work of Rasmussen *et al.* (17). These authors have shown that rat red blood cells respond to isoproterenol with a propranolol-inhibitable increase in cellular cyclic adenosine monophosphate (cAMP) content. Epinephrine and isoproterenol at very low concentrations increased human red cell volume and rendered these cells much more subject to hypotonic hemolysis but did this without a demonstrable increase in cAMP content. This effect in human red cells did not occur in the absence of Ca^{2+} and these same authors have shown that isoproterenol reduces the exchangeable Ca^{2+} pool in human red cells. The data are consistent with a model in which Ca^{2+} rather than cAMP is the "second messenger" in the human red cell's response to epinephrine and isoproterenol. It is not known at this time whether cAMP or Ca^{2+} is more important as a second messenger for that system in rhesus red cells. In either case, several phosphoproteins known to be important in regulation in nucleated cells are both cAMP dependent (ultimately) and Ca^{2+} dependent. Some of these proteins exist in mammalian red cells and might have an effect on glycolysis.

In view of the permeability of red cells to epinephrine (15) and the documented direct effects of catecholamines on red cell size and osmotic fragility (17), it is our working hypothesis that epinephrine affects rhesus red cell glycolysis by acting directly on the erythrocyte. Further, we have found no logical mechanism by which the red cell glycolytic response could be secondary to an epinephrine response in other tissues. However, we can not rigorously exclude the possibility that the red cell response may be secondary in nature. We think this area deserves further study because it may shed additional light on the mechanism of catecholamine action.

Summary. Erythrocyte adenosine triphosphate (ATP) concentration was found to increase in adult rhesus monkeys which have been injected with 3.5 mg of epineph-

rine. The average increase in red cell ATP concentration was 19.5% (significant at the 1% probability level). Epinephrine injection also increased the concentration of the early red cell glycolytic intermediates, glucose 6-phosphate (G6P), fructose 6-phosphate (F6P), and dihydroxyacetone phosphate (DHAP). Respiratory alkalosis, which might have been induced by the epinephrine, is not responsible for the glycolytic changes. This effect of epinephrine on rhesus red cell glycolysis is compatible with an *in vivo* stimulation of red cell hexokinase by epinephrine or some substance elicited by epinephrine and suggests the possibility that such a stimulation may have a physiologic role in the mammalian erythrocyte.

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