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The Effect of Cyanate on the Clotting Proteins and Platelet Function[1] (38732)

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(Introduced by R. L. Engle, Jr.)

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Cyanate, as potassium or sodium salt, when incubated with red cells containing hemoglobin-S will inhibit the sickling phenomenon. Cerami and Manning (3) have shown that cyanate reacts with uncharged amino groups of various proteins in a specific irreversible carbamylation reaction. The preclinical evaluation of cyanate, the effect of this molecule on the clotting process and the further effects of carbamylation on the clotting proteins and platelet function were investigated.

Materials and Methods. Fresh normal blood was drawn in plastic syringes and anticoagulated with buffered-citrate (pH 7.2) in the usual 9:1 concentration and spun at 10,000 rpm for 10 min at 4°. The platelet poor plasma was removed and incubated with freshly prepared sodium cyanate in phosphate buffered saline pH 7.2 to give final concentrations in phosphate buffered saline. The screening tests, Quick (8) prothrombin time (PT), activated partial thromboplastin (PTT) (Procter *et al.* (7)) and thrombin time (TT) as well as assays for Factors I, II, V, VII, VIII, IX, X and XI according to methods compiled by Bowie, Thompson, Didisheim and Owen (2) were done on this plasma after 0, 30 and 60 min incubation at 37.5°.

To determine the effect of pH change on plasma factors, normal plasma was incubated with 100 m*M* NaNCO, 10 m*M* NaNCO and PBS for 1 hr at 37.5°, dialysed against phosphate buffer pH 7.2 and assayed for Factor X.

For the platelet function studies the anticoagulated blood was spun at 900 rpm for 10 min and the platelet rich plasma (PRP) removed. PRP was incubated with sodium cyanate at final concentration of 1 m*M*, 10 m*M* and 100 m*M*. Platelet factor 3 release was measured using the Kaolin clotting time of Hardisty (6) after 0, 30 and 60 min of function. Platelet numbers were matched in control and test samples. The platelet factor 3 release was repeated with platelets washed after incubation with cyanate and resuspended in fresh normal plasma.

Aggregation of 0.4 ml. platelet rich plasma incubated with sodium cyanate in final concentrations of 10 m*M* and 100 m*M* was followed in a chronolog aggregometer (Chronolog Corporation) using epinephrine (final concentration 5.5×10^{-4} *M*), ADP (final concentration 2×10^{-4} *M*) and collagen (suspension of calf tendon in saline) as the inducing agents. Platelet rich plasma was diluted to provide platelet counts of approximately 300,000/mm^3 before the additives were introduced. Percent aggregation was calculated with the following formula:

$$\% \text{ aggregation} = 1 - \frac{\text{O.D. max.}}{\text{O.D. initial}} \times \text{K},$$

where K = 112.49859 as modified from the formula of Corby *et al.* (5).

Since the epsilon amino groups of lysine may be carbamylated by cyanate and because this amino acid may be the active site of collagen–platelet interaction (Wilner *et al.* (9)) the effect of cyanate on collagen was tested in a final experiment. Collagen in final concentrations of 100 mM and 1 mM was incubated at 37.5° for 1 hr with 1 *M* NaNCO, 0.1 *M* NaNCO and PBS as control, dialysed back to pH 7.2 and tested for ability to induce aggregation of normal platelet rich plasma in the Chronolog aggregometer. Percent aggregation was calculated as given above.

Results. The results of the screening tests, PT, PTT and TT are shown in Fig. 1. An immediate rise in the clotting times may be seen in the PT and PTT over controls which

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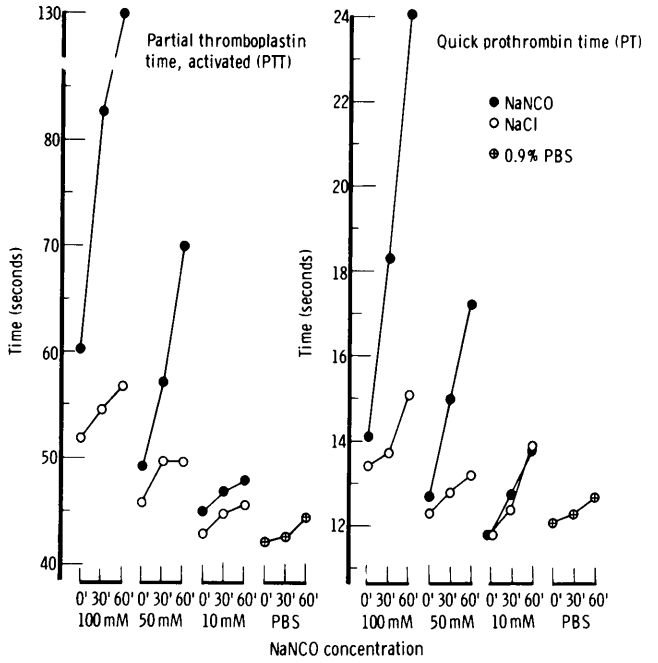

FIG. 1. Screening tests: Prothrombin time, Partial Thomboplastin Time when incubated with cyanate.

progressed with time, the greatest changes occurring with the 100 m*M* additive. No immediate changes were seen in the amount of thrombin generated in the TT (Table I). However, an 80% inhibition of thrombin generation was seen after 60 min incubation with 100 m*M* NaNCO and 46% inhibition with 50 m*M* additive. No inhibitory effects were seen with the 10 m*M* additive.

Factor assays revealed no significant immediate change or significant change with incubation in the levels of Factor I. A maximum decrease of 7% of control occurred in Factor I levels with the 100 m*M* additive and no changes with the lesser concentrations were observed. Factor VIII levels decreased 25% compared to the control (PBS) levels at zero time in the presence of 100 m*M* cyanate or saline additive. A 14% decrease in zero time values was noted with the 50 m*M* and 10 m*M* additives. A minimal increase in assay levels was observed with time. Minor changes were seen with incubation in the Factor II levels which improved with time, the greatest effect occurring with the 100 m*M* concentration. These changes occurred with equivalent concentrations of NaCL and were not due to the cyanate alone. A similar pattern was seen in the levels of Factor V (Table I).

Greater changes were seen with Factors VII, IX, X and XI, which could be attributed to carbamylation of the proteins and not to the effect of NaCl. Immediate zero time effects were minimal and were also present in the NaCl controls except for Factor X. Changes induced by incubation were significant with 63–71% inhibition of factor assay levels with time. The assay levels ranged between 29–38% of the PBS control after 60 min incubation with 100 m*M* NaNCO. These changes were seen to a lesser degree with the lower concentrations (Table I).

The effect of pH change as measured by Factor X activity was noted only with 100 m*M* NaNCO at both zero time and after 60′ incubation (Table II). The effect of cyanate on Factor X activity was the same as that in nondialyzed plasma (Table I).

The effect of carbamylation of collagen by cyanate at 100 m*M* and 1 m*M* concentration on the ability of collagen to induce aggregation was insignificant. Percent aggregation produced by normal collagen and collagen incubated with cyanate was the same.

The aggregation of platelets induced by ADP was normal when compared with the NaCl control when epinephrine and col-

TABLE I. CLOTTING FACTOR ACTIVITY

Factor		Clotting factor activity (% of control 0.9% PBS) 0′	30′	60′
II	100 m*M*	101	82	95
	50 m*M*	117	88	102
	10 m*M*	117	100	102
V	100 m*M*	80	61	78
	50 m*M*	85	72	78
	10 m*M*	90	82	90
VII	100 m*M*	98	99	38
	50 m*M*	98	80	70
	10 m*M*	99	105	100
IX	100 m*M*	95	58	31
	50 m*M*	99	64	45
	10 m*M*	105	90	75
X	100 m*M*	85	47	29
	50 m*M*	88	68	54
	10 m*M*	102	93	80
XI	100 m*M*	87	55	38
	50 m*M*	90	81	58
	10 m*M*	108	105	101
II_a (thrombin)	100 m*M*	80	51	20
	50 m*M*	77	71	51
	10 m*M*	81	78	74

lagen were used as aggregation inducing agents in the presence of 100 m*M* NaNCO, platelet aggregation was grossly abnormal and partially corrected by resuspension in fresh normal plasma. Lesser changes were noted with 10 m*M* NaNCO (Fig. 2).

Platelet function studies revealed an abnormality in the Factor 3 release only after 60′ incubation with 100 m*M* NaNCO. This apparent abnormality of platelet function was corrected by washing and resuspending the "carbamylated" platelets in fresh normal plasma ($n = 4$) (Table III).

Discussion. Cyanate is known to react with the amino terminal groups and epsilon amino groups of many proteins in the whole animal. Cerami *et al.* (4) have shown that 7.5% of dose 14-C labeled cyanate injected into mice combined with the amino terminal valine of the hemoglobin molecule; 70–75% was broken down to carbon dioxide, a large percentage of the remaining compound went to muscle and bone, and a small amount to other tissues. This carbamylation is irreversible. Therefore, it was deemed necessary to investigate the effect of this compound on the clotting proteins before embarking on a large clinical trial in patients with sickle cell disease. The concentrations used in these studies were below and above the concentrations used in patients but were within the range capable of inhibiting the sickling phenomenon *in vitro*.

TABLE II. FACTOR × CONCENTRATION[a]

	0′	60′
0.9% P.B.S.	96%	100%
10 m*M* NaNCO	100%	105%
100 m*M* NaNCO	64%	16%

[a] pH effect of incubation of cyanate 100 m*M* on plasma using the concentration of Factor × as the end point. Normal plasma diluted 9:10 P.B.S.

An immediate effect on the clotting proteins was present that was not related to a pH effect as demonstrated by the Factor X assay before and after dialysis against phosphate buffer at pH 7.2. This small change was reflected in the factor V and X zero time assays of 7 and 15% respectively with the 100 m*M* additive only. The inhibitory effects of carbamylation increased with time and was reflected in all of the screening tests, the PT, PTT and TT. The specific 80% inhibition of thrombin and the 63–75% inhibition of Factors VII, IX and XI at the highest concentration of additive accounts for the changes in both PT and PTT. The lack of effect on fibrinogen was not seen by Bell and Charache (1) who reported a decrease in clottable protein in fibrinogen solution and plasma at a high (150 m*M*) concentration of NaNCO. They reported no change in the amount of antigenic material present. They also found a minimal immediate effect of 100 m*M* NaNCO on thrombin and inhibition was unchanged with prolonged incubation (2 hr).

Although Cerami[3] did not find carbamylation of the epsilon amino groups of lysine at 10 m*M* cyanate concentration, the possibility that carbamylation might occur at higher concentrations was considered. Since the terminal lysine of collagen is thought to be the active site of platelet adhesion and aggregation (Wilner *et al.* (9)), this important and potentially adverse effect of higher cyanate concentrations on collagen induced platelet aggregation was investigated. The

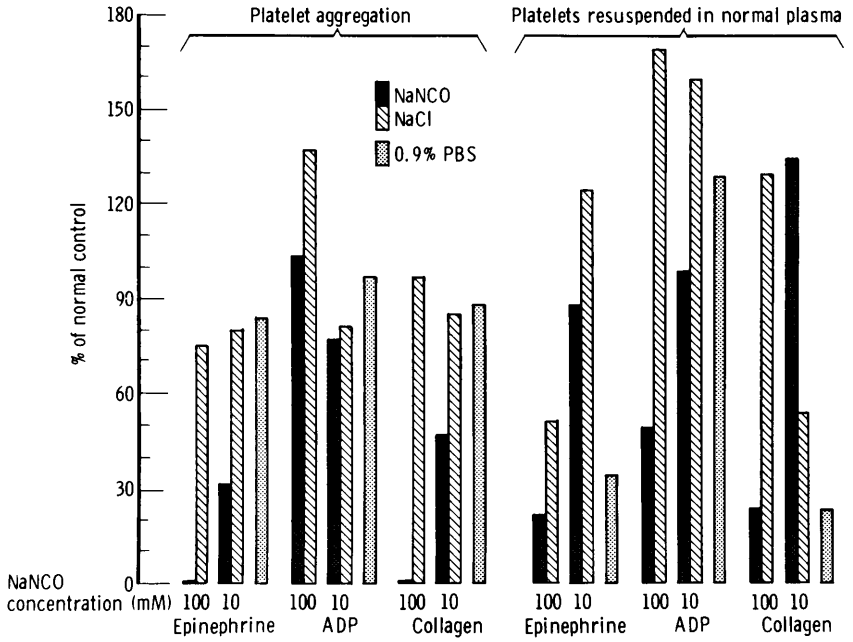

FIG. 2. Aggregation studies using platelet rich plasma and inducing agents as noted when incubated with cyanate.

TABLE III. PLATELET FACTOR 3 RELEASE[a]

	0′		60′	
	Carbamylated platelets and plasma	Washed carbamylated platelets and normal plasma	Carbamylated platelets and plasma	Washed carbamylated platelets and normal plasma
0.9% PBS	46.1 sec	38.2 sec	54.7 sec	37.8 sec
100 m*M* NaNCO	51.4	37.6	80.8	37.6
10 m*M* NaNCO	46.7	39.1	52.3	42.0
100 m*M* NaCl	51.5	37.3	48.9	38.6
10 m*M* NaCl	44.7	38.4	55.2	44.4

[a] Platelet Factor 3 Release as measured by the Kaolin Clotting Time showing correction of abnormality with washing of carbamylated platelets and resuspension in normal plasma. Values represent mean of four experiments. Additives are given in final concentrations.

results indicate no inhibition of aggregation and therefore no carbamylation of the e-amino group.

The platelet release of factor 3 was normal; the abnormal results appeared in the test system due to carbamylation of the plasma clotting proteins. Aggregation was abnormal only at high concentrations of cyanate and was partially reversible by resuspending platelets in normal plasma. Neither immediate changes nor carbamylation effect noted *in vitro* have been seen in the clotting function of the few initial patients to whom sodium cyanate has been given orally. This suggests that the turnover rate of all of the clotting proteins may be sufficiently high to compensate for the initial binding seen in vitro and protect the patients from further carbamylation changes. Work is in progress to prove this hypothesis.

The observed insignificant effect upon coagulation proteins in vitro should not

preclude the use of this agent in the proposed clinical trials in patients with sickle cell disease.

Summary. Cyanate reacts with unchanged amino groups of various proteins in a specific irreversible carbamylation reaction. The effect of this molecule on the clotting process and the effects of carbamylation on the clotting proteins and platelet functions were investigated in vitro. An immediate effect on the clotting proteins, not related to pH, was seen in the screening tests prothrombin time, partial thromboplastin time and thrombin time at the highest concentration (100 m*M*), to a lesser degree at the lower concentration (10 m*M*). These changes reflected decreases of 19 and 36% respectively in Factor V and X activity, an inhibition of 63–75% of Factors VII, IX, X and XI activity, and 80% inhibition of thrombin activity. The inhibitory changes of carbamylation increased with time. No changes were seen in the activity of Factors I and VIII.

Platelet function studies revealed no inhibition of Factor III release; aggregation was abnormal only at high concentrations with epinephrine and collagen induction and partially reversible by resuspension in normal plasma.

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