The Cobalamin Precursor Cobinamide Detoxifies Nitroprusside-Generated Cyanide

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Sodium nitroprusside is used to treat hypertensive emergencies and acute heart failure. It acts by releasing nitric oxide (NO), a highly potent vasodilator, but unfortunately, for each NO molecule released, five cyanide ions are released. Thus, nitroprusside therapy is limited by cyanide toxicity. Therefore, a cyanide scavenger could be beneficial when administering nitroprusside. Hydroxocobalamin, which has a relatively high binding affinity for cyanide, has been shown to reduce cyanide levels in nitroprusside-treated patients. Cobinamide, the penultimate precursor in hydroxocobalamin biosynthesis, has a much greater affinity for cyanide than cobalamin, and binds two cyanide ions. We now show that cobinamide is highly effective in neutralizing cyanide ions released by nitroprusside in cultured mammalian cells, *Drosophila melanogaster*, and mice. Cobinamide also binds NO, but at molar concentrations 2.5–5 times that of nitroprusside, it did not decrease NO concentrations or the physiological effectiveness of nitroprusside. We conclude that cobinamide could be a valuable adjunct to nitroprusside therapy. Exp Biol Med 232:789–798, 2007

Key words: acute hypertension; nitroprusside; nitric oxide; cyanide; cobinamide

Introduction

Sodium nitroprusside (disodium pentacyanonitrosylferrate [III] dihydrate) has a long history of clinical use, having been described in 1883 as an agent that could detect ketone bodies in the urine of diabetics (1). It was recognized as a hypotensive agent in 1887, and the first clinical trial of nitroprusside was in 1928 (1). The work of Page *et al*. in the early 1950s popularized intravenous nitroprusside as an antihypertensive agent, and it was the mainstay for treating acute hypertensive emergencies for many years (2). Although other agents are now available for treating hypertensive emergencies, most current reviews on this topic list nitroprusside as a first-line drug, and because of its arterial and venous dilating properties, it is also used to treat acute heart failure syndromes (3–5). Over one million 50 mg-dose vials of nitroprusside were sold in the United States in 2005.[1]

Nitroprusside acts by rapidly releasing nitric oxide (NO), an extremely potent vasodilator (6). *In vivo*, NO has a very short half-life, on the order of seconds to minutes (7). Thus, a major advantage of nitroprusside is that it can be quickly titrated to the needs of the patient. A major disadvantage of nitroprusside, however, is that five cyanide ions are released for every NO molecule generated. Cyanide potently inhibits mitochondrial cytochrome *c* oxidase, thereby arresting oxidative phosphorylation and depleting cellular ATP (8). Cyanide is detoxified by the mitochondrial enzyme rhodanese, which uses thiosulfates to convert cyanide to thiocyanate, a relatively nontoxic compound (8). This endogenous cyanide detoxification mechanism is sufficient when nitroprusside is infused at low rates for short periods, but over time, thiosulfates are exhausted and cyanide can accumulate to toxic levels (2). Several deaths have been attributed to cyanide toxicity in nitroprusside-treated patients, and currently serum thiocyanate concentrations are monitored closely in patients receiving nitroprusside (9–11). Cyanide toxicity is of particular concern in patients with hepatic and renal disease, since rhodanese is

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[1] Intercontinental Marketing Services (http://www.imshealth.com).

present predominantly in the liver, and cyanide and thiocyanate are excreted by the kidneys.

Sodium thiosulfate and nitrites—amyl nitrite and sodium nitrite—are approved in the United States for treating cyanide toxicity, and some authors recommend co-administering sodium thiosulfate with nitroprusside (2, 9, 12). Sodium thiosulfate can cause nausea, vomiting, and diarrhea, and nitrites generate methemoglobin and can induce hypotension; thus, these agents are not ideal for reducing cyanide toxicity associated with nitroprusside therapy (8). Hydroxocobalamin (vitamin B_{12a}) binds cyanide with high affinity ($K_A \sim 10^{12}\ M^{-1}$), and several studies have shown it reduces cyanide toxicity in nitroprusside-treated patients (13–15). Cobinamide, the penultimate compound in cobalamin biosynthesis, binds two cyanide ions, the first one with at least 100 times higher affinity than hydroxocobalamin (i.e., $K_A \sim 10^{14}\ M^{-1}$). Previous research by the authors has shown that cobinamide is superior to hydroxocobalamin as a cyanide detoxifying agent, both in cultured cells and in a *Drosophila melanogaster* model of cyanide toxicity (16). Cobinamide also binds NO, but much less tightly than cyanide (K_A for NO $\sim 10^{10}\ M^{-1}$), and once a cyanide molecule is bound to cobinamide, it can no longer bind NO (17). These data led us to hypothesize that cobinamide, at appropriate concentrations, should detoxify nitroprusside-generated cyanide without reducing NO concentrations. We now show this hypothesis to be correct in vascular smooth muscle cells, *D. melanogaster*, and mice, and thus cobinamide might be a good agent to ameliorate cyanide toxicity in nitroprusside-treated patients.

Materials and Methods

Materials. CS-54 rat pulmonary artery smooth muscle cells (A. Rothman, University of California at San Diego) were cultured as described previously; they were used at passage five and maintain differentiated properties through multiple subcultures (18, 19). Canton-S wild-type *D. melanogaster* (Bloomington Stock, Bloomington, IN) and male C57BL/6J mice, ages 10–12 weeks (Jackson Laboratories, Bar Harbor, ME) were used in the study. Mice were fed Teklad 7001 standard diet (Harlan, Madison, WI) *ad libitum*, and the mouse studies were performed according to NIH Guidelines for the Care and Use of Laboratory Animals approved by the Institutional Animal Care and Use Committee of the Veterans Administration San Diego Healthcare System. Sodium nitroprusside (Sigma Chemical Co., St. Louis, MO) was made fresh on the day of use and shielded from light. Aquahydroxocobinamide, referred to throughout the text as cobinamide, was prepared from hydroxocobalamin (Wockhardt Ltd., Mumbai, India) as described previously; by several criteria, it was greater than 95% pure (20). The guanylate cyclase inhibitor 1H-(1,2,4)oxadiazolo(4,3-a)quinoxalin-1-one (ODQ) was from Calbiochem, La Jolla, CA. Antibodies against vasodilator-stimulated phosphoprotein (VASP) phosphorylated on serine 239 and against actin were from NanoTools (Teningen, Germany) and Santa Cruz Biotechnology (Santa Cruz, CA), respectively. Beveled 33-gauge needles and 2.5-μl syringes were from Hamilton Company (Reno, NV). An automated, computer-controlled noninvasive blood pressure analysis system for rodents (SC1000 Hardware with SC1000 Comm Software) was from Hatteras Instruments (Cary, NC).

Calculations of Free NO Concentrations in the Presence of Cobinamide. For cobinamide to be useful as an adjunct to nitroprusside therapy, it would need to decrease cyanide concentrations without decreasing NO concentrations. To evaluate if this is possible, we used the COMICS program, which calculates the concentration of each species of a ligand binding partner interaction, knowing the relevant affinity constants (21). These calculations assumed (i) full dissociation of nitroprusside, (ii) nitroprusside releasing five cyanide ions for every NO molecule, and (iii) cobinamide binding two cyanide ions.

Measurement of Respiratory Activity of CS-54 Cells. Mitochondrial respiratory activity was assessed by measuring oxygen consumption using a Clark electrode as described previously (16). Briefly, CS-54 cells were permeabilized with digitonin, suspended in a Hepes-based buffer, and placed in a metabolic chamber maintained at 37°C. After measuring basal oxygen consumption for 5 mins, 5 m*M* sodium succinate and 5 m*M* glycerol 3-phosphate were added to stimulate mitochondrial respiration. Nitroprusside at a final concentration of 10 m*M* was then added, followed by cobinamide at 250 μ*M*.

Measurement of Nitrite and Nitrate in CS-54 Cells and *D. melanogaster* In the presence of oxygen, NO is converted rapidly to two stable products, nitrite and nitrate, and measuring these two NO oxidation products is commonly done to assess NO production (22). We measured nitrite and nitrate using a Griess reagent-based kit from Active Motif as described previously (20). Measurements were performed on culture medium of CS-54 cells incubated in phenol red-free medium, and in extracts of decapitated flies; phenol red-free medium and fly decapitation (to eliminate eye pigment) were necessary to maintain a low background in the assay.

Assessment of VASP Phosphorylation in CS-54 Cells. VASP is an important regulator of actin dynamics, and is phosphorylated in endothelial and smooth muscle cells in response to NO and other vasodilators (23). By activating guanylate cyclase, NO increases intracellular cGMP, which then activates cGMP-dependent protein kinase. The latter enzyme phosphorylates VASP on Ser239 preferentially, but also on Ser157, and assessing Ser239 phosphorylation provides a measure of NO generation (24). We assessed VASP Ser239 phosphorylation using a phospho-specific antibody, as described previously (25). Briefly, CS-54 cells were treated with varying concentrations of nitroprusside for 1 hr and were extracted directly into a hot sodium dodecyl sulfate (SDS)-

based buffer; the extracts were subjected to SDS polyacrylamide gel electrophoresis followed by Western blotting. Half of each sample was analyzed by the antiphosphoserine 239 antibody and half by an antiactin antibody.

Injection of Flies with Nitroprusside and Exposure of Flies to Cyanide. Flies were injected with drugs as described previously (16, 26). Briefly, flies were anesthetized on ice, and injected into the thorax with 1 μl of fluid using a 33-gauge needle. Greater than 95% of flies survived the injection, and injecting water had no ill effects. Flies were exposed to 22 ppm cyanide gas generated from KCN as described previously (16).

Measurement of ATP in Fly and Malpighian Tubule Extracts. ATP was measured as described previously by the luciferase-luciferin method (27). Briefly, decapitated flies or isolated Malpighian tubules (isolated as described below) were extracted rapidly in ice-cold perchloric acid, and the extracts were neutralized with $KHCO_3$. After adding luciferase and luciferin to the extracts, ATP was measured in a luminometer and quantified by comparison to a standard curve.

Measurement of Fluid Secretion by Malpighian Tubules. Malpighian tubules of *D. melanogaster* are the insects' fluid and osmoregulatory organ, corresponding to vertebrate kidneys. We isolated tubules and measured rates of fluid secretion across tubular cells as described previously (16, 20). Briefly, the two pairs of Malpighian tubules of a fly were resected and suspended in mineral oil; the ureteral end of the tubule was immobilized on a dissecting pin and the opposite end of the tubule was bathed in 10 μl of Schneider's insect medium. The amount of fluid transported by the tubules was determined every 10 mins by measuring the size of a drop formed at the end of the ureter. Tubular secretion is stimulated by adding secretagogues, such as NO, to the Schneider's medium (20).

Measurement of *D. melanogaster* Heart Rate. We measured heart rates of *D. melanogaster* as described previously (26, 28). Injecting flies with 1 μl of fluid induces tachycardia, and 4 hrs is the minimum time required for the heart rate to return to that of uninjected flies; therefore, all measurements were performed 4 hrs postinjection. Flies were anesthetized with 50% triethylamine (FlyNap), which we have shown has no effect on heart rate, and immediately thereafter were placed on a microscope slide. An inverted microscope was used to record a 20-sec moving image of each fly heart, and a two-dimensional time-space, or M-mode, representation of cardiac contraction was generated from image intensities measured along a line perpendicular to the heart tube. The frequency of contraction was derived from the average intensity of pixels on the line. A beat detection algorithm was used to count the number of systolic contractions by detecting peaks within this average intensity signal (28, 29).

Measurement of Mouse Blood Pressure. Pulse and systolic and diastolic blood pressures were measured non-invasively in the tail artery of mice by restraining the mice on a warming platform maintained at approximately 38°C. The base of the mouse's tail was placed in a computer-controlled pneumatic cuff, and the distal tail was placed in a sensor assembly consisting of a light emitting diode and a photodiode detector. Baseline measurements were made with the mice awake in a dark chamber. The mice were then anesthetized with 3% isoflurane in an induction chamber, and returned to the platform where they received 1.5% isoflurane with 2 liter/min of oxygen via a nose cone. Baseline anesthetized measurements were made, after which the mice were injected intraperitoneally with 1 μmol cobinamide in 70 μl of buffer; 30 mins later, the mice received 0.4 μmol sodium nitroprusside in 100 μl of buffer intraperitoneally. Blood pressure and pulse were then measured every 30 secs for 15 mins.

One hour after completing the blood pressure measurements, the mice were euthanized and the chest and abdomen were opened; blood was aspirated from the right ventricle and urine was aspirated from the bladder. Samples were placed immediately on ice, and after 1 hr, the blood was subjected to centrifugation to separate serum and cells. Samples were kept at 4°C in capped syringes until analyzed.

Measurement of Mouse Serum and Urinary Thiocyanate Concentrations. Thiocyanate in the serum and urine was oxidized at 37°C to cyanide using acidified $KMnO_4$ (30). The resulting HCN gas was collected in KOH, and measured by the spectrophotometric method of Guilbault and Kramer, as modified by Gewitz *et al*. (31, 32). The assay is highly reproducible and specific, with a sensitivity of 50 pmol KCN. The assay also measures cyanide but, at physiological pH and temperature, cyanide exists as HCN gas, which is volatile. Thus, without taking special precautions, it is unlikely that cyanide was present in the samples, but a small amount of cyanide would not interfere with the data because it is the precursor of thiocyanate.

Data Analysis. Comparisons between two sets of data were analyzed using a one-tailed *t* test, and comparisons among three or more sets of data were analyzed by analysis of variance. In both cases, $P < 0.05$ was considered statistically significant.

Results

Calculation of NO and Cyanide Ion Concentrations, at Varying Ratios of Nitroprusside and Cobinamide. Using the COMICS program (21), we calculated that at equimolar concentrations of cobinamide and nitroprusside, cobinamide will reduce the cyanide concentration by 40% and will have no effect on the NO concentration (Table 1; the nitroprusside concentration was set at 1 μ*M*, but similar results were obtained at any nitroprusside concentration). As the cobinamide concentration is increased to 2.5 times that of nitroprusside, the cyanide concentration will be reduced by 97% and the NO

Table 1. Calculation of Free and Bound NO and Cyanide Ion Concentrations at a Constant Nitroprusside Concentration and Varying Cobinamide Concentrations[a]

Cbi	CbiCN (μM)	$Cbi(CN)_2$ (μM)	CbiNO (μM)	Free CN (μM)	Free NO (μM)
None	—	—	—	5	1
1 μM	3.3×10^{-3}	0.997	1.4×10^{-6}	3	1
2.5 μM	0.15	2.35	1.3×10^{-4}	0.15	1
5 μM	4.25	0.38	0.38	8.0×10^{-4}	0.62

[a] Cbi, cobinamide; CN, cyanide; NO, nitric oxide. Calculations were made using the COMICS program, which calculates free and bound ligand concentrations when the binding affinities for ligands (in this case NO and CN ion) to their binding partner (Cbi) are known (21). The nitroprusside concentration was kept constant at 1 μM, yielding a CN concentration of 5 μM, and the Cbi concentration was varied as indicated.

concentration will remain unaffected (Table 1). When a cobinamide concentration 5 times that of nitroprusside is reached, free cyanide is effectively eliminated, and the NO concentration will be decreased by about 40% (Table 1). These calculations indicate that reasonable working concentrations of cobinamide would be 2 to 5 times that of nitroprusside. In the following sections, we tested the validity of these calculations in cultured cells, *D. melanogaster*, and a mouse model.

Cobinamide Recovers Respiratory Activity in Nitroprusside-treated CS-54 Cells but Does Not Reduce NO Concentrations. Mitochondrial cytochrome *c* oxidase activity can be assessed by measuring respiratory activity of permeabilized cells incubated with succinate and glycerol-3 phosphate (33). Since cyanide is a rapid and potent inhibitor of cytochrome *c* oxidase, nitroprusside would be expected to decrease respiratory activity of cells. We found that treating CS-54 rat vascular smooth muscle cells with 10 m*M* nitroprusside rapidly decreased cellular oxygen consumption (Fig. 1a; left half of panel a shows a representative tracing, and right half of panel a shows a summary of three independent experiments). Providing 250 μM cobinamide to the cells almost fully recovered respiratory activity to pre-nitroprusside levels (Fig. 1a; cobinamide alone had no effect on respiratory activity). Under the conditions of the assay, only about 2% of the nitroprusside decomposed to NO and cyanide as determined by measuring cyanide and nitrite and nitrate (data not shown); this explains why only a relatively small amount of cobinamide was required to neutralize the cyanide released from nitroprusside. *In vivo*, nitroprusside decomposition is considerably faster because of reducing compounds such as glutathione and ascorbic acid (2); these could not be added to the system because they interfered with measurement of respiratory activity.

Since the calculations in Table 1 suggest that cobinamide at a concentration 2.5 times that of nitroprusside should not reduce nitroprusside-generated NO, we performed two sets of experiments in CS-54 cells to test this premise. First, we measured nitrite and nitrate in the medium of CS-54 cells incubated with nitroprusside; under physiological conditions, NO is oxidized rapidly to nitrite and nitrate, and hence the latter can be used to assess NO concentrations (19). As we found previously (20), CS-54 cells produce a significant amount of NO under basal nonstimulated conditions (Fig. 1b, left half of figure). Treating the cells with 10 μM nitroprusside increased the amount of nitrite and nitrate in the medium, and adding cobinamide at 2.5 times the nitroprusside concentration (i.e., 25 μM) actually increased nitrite and nitrate concentrations further (Fig. 1b, right half of figure). The increased nitrite and nitrate in the presence of cobinamide could have been from cobinamide stimulating nitroprusside decomposition by binding cyanide and shifting the equilibrium to more nitroprusside in the dissociated state; this would not likely happen in a clinical setting because, as mentioned above, nitroprusside decomposition is fast *in vivo*. In the second set of experiments, we assessed cGMP-dependent protein kinase phosphorylation of VASP (23). By activating guanylate cyclase and increasing cGMP concentrations, NO induces VASP phosphorylation on at least two sites: serines 157 and 239 (24). In the basal nonstimulated state, phosphorylated VASP could not be detected using an antibody directed against phosphoserine 239, but adding 3 μM nitroprusside to cells induced strong phosphorylation of VASP (Fig. 1c; two bands of phosphorylated VASP appear because VASP is also phosphorylated on serine 157, and

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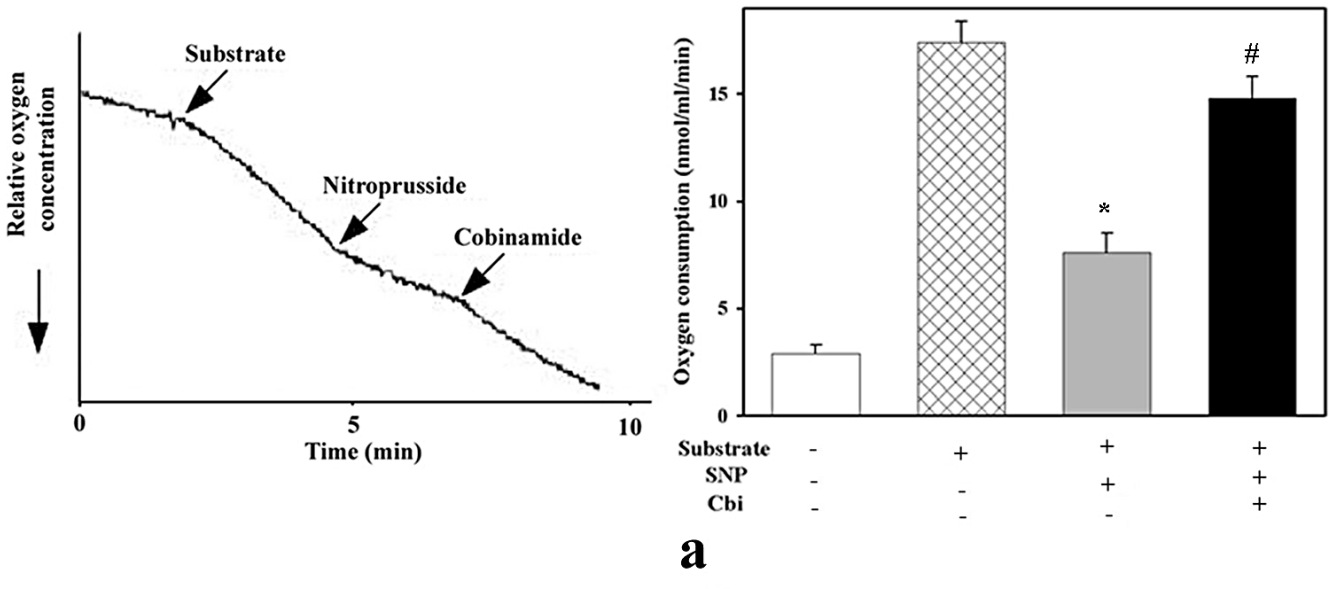
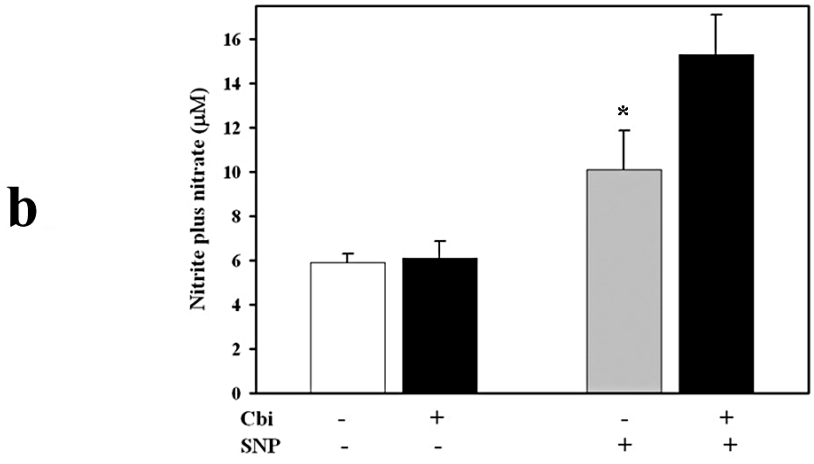
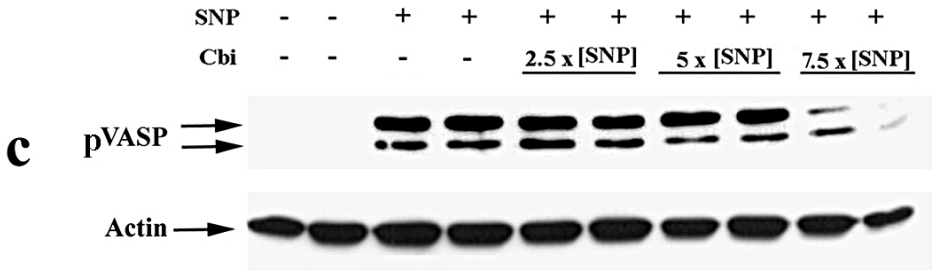
Figure 1. Effect of cobinamide on respiratory activity and NO concentrations in nitroprusside-treated CS-54 cells. (a) CS-54 rat pulmonary artery smooth muscle cells were harvested, permeabilized with digitonin, and placed in a metabolic chamber as described in Methods. Respiratory activity was assessed by measuring oxygen consumption over time using a Clark electrode. Left half of panel shows a representative tracing; right half of panel shows oxygen consumption calculated by determining the slope of the line of three independent experiments (mean ± SD). Under basal nonstimulated conditions, oxygen consumption was low (open bar and note relatively shallow slope in the tracing). Adding substrate (5 m*M* sodium succinate and 5 m*M* glycerol 3-phosphate) increased oxygen consumption about six-fold (cross-hatched bar and increased slope in the tracing). Adding 10 m*M* nitroprusside (SNP) to the substrate-stimulated cells reduced oxygen consumption by more than 50% (gray bar), while adding 250 μM cobinamide (Cbi) to the substrate- and nitroprusside-treated cells returned oxygen consumption almost to pre-nitroprusside levels (black bar). The inhibition of respiratory activity by nitroprusside was statistically







significant (* $P < 0.01$ for the comparison of substrate alone to substrate plus nitroprusside), as was the recovery by cobinamide (# $P < 0.05$ for the comparison of nitroprusside to nitroprusside plus cobinamide). (b and c) CS-54 cells were incubated for 1 hr in the absence or presence of 10 μM nitroprusside (SNP) and 25 μM cobinamide (Cbi) (b), or 3 μM nitroprusside and 7.5 μM (2.5 $\times$ SNP), 15 μM (5 $\times$ SNP), or 22.5 μM (7.5 $\times$ SNP) cobinamide (c). Nitrite and nitrate were measured in the culture medium using an enhanced Griess reagent (b), and VASP phosphorylation was assessed using an antibody specific for VASP phosphorylation on serine 239 (c, pVASP). The upper VASP band in panel c represents VASP phosphorylated on serine 239 and serine 157, while the lower band represents phosphorylation on serine 239 only. Actin was used as a loading control because several antibodies that recognize total nonphosphorylated VASP were of poor quality. The data in panel b are the mean $\pm$ SD of three independent experiments performed in duplicate, and the experiments in panel c were repeated at least two additional times with similar results. The increase in nitrite and nitrate by nitroprusside was significantly different (* $P < 0.05$), but there was no statistical difference between nitroprusside and nitroprusside plus cobinamide.

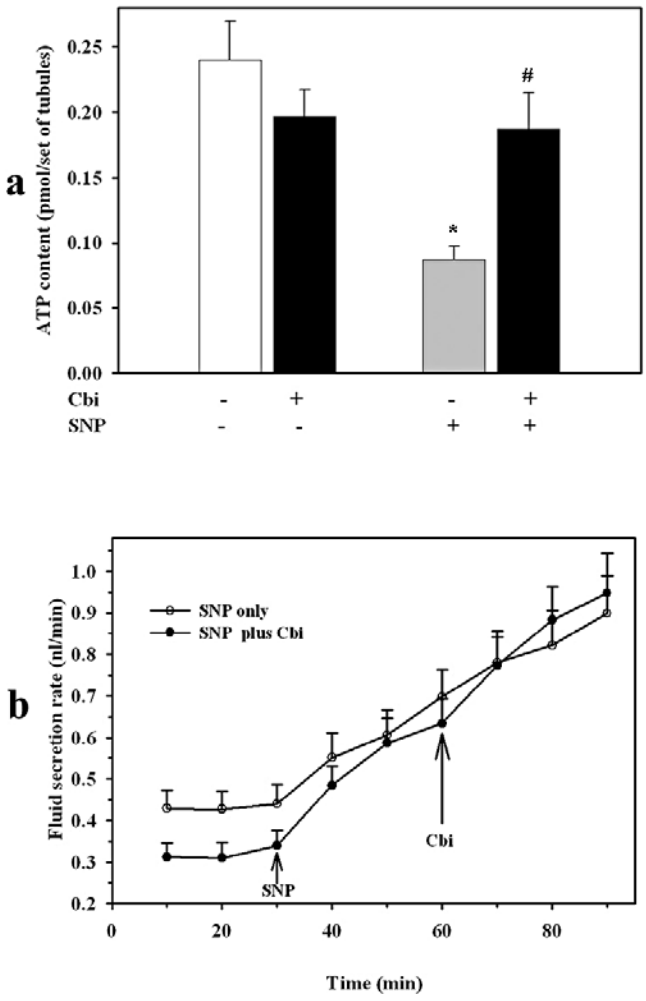


Figure 2. Effect of cobinamide on ATP concentrations, and NO-dependent fluid secretion in *D. melanogaster* Malpighian tubules treated with nitroprusside. Malpighian tubules were resected from *D. melanogaster*, and treated *ex vivo* with 50 μ*M* nitroprusside (SNP) for 1 hr; as indicated by the short arrow in (b), the nitroprusside was added after a 30-min equilibration period. Some tubules additionally received cobinamide (Cbi), either simultaneously with the nitroprusside (black bars, a) or 30 mins after the nitroprusside (long arrow, b, filled circles). ATP content of the tubules (a) and fluid secretion rates (b) were measured as described in Materials and Methods. The data are the mean ± SD of at least three independent experiments performed in duplicate. The decrease in ATP content by cobinamide in control cells was not significant, whereas the decrease in ATP by nitroprusside was significant (* $P < 0.01$), as was the increase in ATP by cobinamide in nitroprusside-treated cells (# $P < 0.01$).

phosphorylation of this latter site shifts VASP to a slower migrating form). Lower nitroprusside concentrations induced proportionately less VASP phosphorylation. Adding cobinamide at a concentration 2.5 times that of nitroprusside (i.e., 7.5 μ*M*) did not affect VASP phosphorylation, whereas 15 μ*M* cobinamide slightly decreased VASP phosphorylation (Fig. 1c). Higher cobinamide concentrations significantly decreased VASP phosphorylation (Fig. 1c; shown is 22.5 μ*M* cobinamide). The modest decrease in VASP phosphorylation at a 5:1 molar ratio of cobinamide to nitroprusside was less than the COMICS program predicted; this discrepancy is considered in the Discussion.

Cobinamide Restores ATP in *D. melanogaster* Malpighian Tubules Treated with Nitroprusside Without Reducing NO-Dependent Fluid Secretion. Malpighian tubules are an insect's major fluid regulatory organ, and fluid secretion is stimulated by NO. Treating isolated *D. melanogaster* Malpighian tubules with 50 μ*M* nitroprusside reduced intracellular ATP concentrations by >60%, presumably via cyanide inhibition of cytochrome *c* oxidase (Fig. 2a); providing 250 μ*M* cobinamide along with the nitroprusside prevented the drop in ATP (Fig. 2a). Tubule secretion was stimulated immediately on adding 50 μ*M* nitroprusside to isolated tubules (Fig. 2b). Providing 250 μ*M* cobinamide to nitroprusside-treated tubules did not significantly change the rate of fluid secretion. Thus, at a concentration five times that of nitroprusside, cobinamide did not interfere with a physiological effect of nitroprusside-generated NO and prevented the toxic effects of coreleased cyanide.

Cobinamide Restores ATP in Nitroprusside-Treated *D. melanogaster* Without Decreasing NO Concentrations. To determine if cobinamide can reverse the toxic effects of nitroprusside-released cyanide in a whole organism, we injected nitroprusside into *D. melanogaster* and measured ATP concentrations in extracts of decapitated flies. We found that injecting 1 μl of 50 μ*M* nitroprusside into flies reduced ATP concentrations by ~50% (Fig. 3a); the fluid volume of *D. melanogaster* is about 10 μl, yielding a nitroprusside concentration of about 5 μ*M* in the flies. Coinjecting 250 μ*M* cobinamide with the nitroprusside recovered ATP concentrations (Fig. 3a; values in control flies and flies that received nitroprusside and cobinamide were not significantly different). For comparison, we exposed flies to a sublethal dose of cyanide and found a similar decrease in ATP and recovery by cobinamide (Fig. 3a).

To determine if cobinamide decreased NO concentrations in the flies, we measured nitrite and nitrate; we found no change in the concentration of these two NO metabolites (Fig. 3B). Once again, the discrepancy between the COMICS program predicting that a 5:1 molar ratio of cobinamide to nitroprusside would decrease the NO concentration, and the lack of an observed effect, will be considered in the Discussion.

Effect of Cobinamide on Nitroprusside Reduction of Heart Rate in *D. melanogaster*. To more closely approximate nitroprusside use in humans, we assessed the effect of nitroprusside on cardiovascular function in flies. We have shown previously that NO reduces the heart rate of *D. melanogaster* (26), and we found that injecting 1 μl of 50 μ*M* nitroprusside decreased the heart rate of flies by ~45% (Fig. 4). Coincident with the reduction in heart rate, nitroprusside-injected flies ceased flying and became listless. The nitroprusside-induced reduction in heart rate and physical activity appeared to be from nitroprusside's

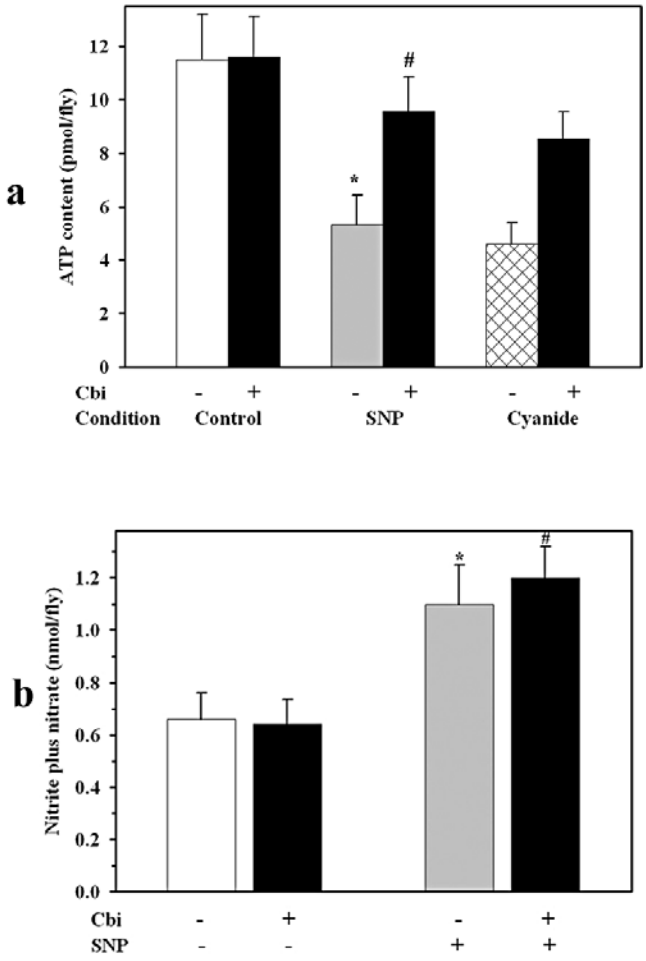


Figure 3. Effect of cobinamide on ATP, and nitrite and nitrate concentrations in nitroprusside-treated *D. melanogaster*. *D. melanogaster* were injected with 1 μl of water (open bars) or 1 μl of 50 μ*M* nitroprusside (SNP) (gray bars); some flies were coinjected with 250 μ*M* cobinamide (Cbi) (black bars). One hour later, the flies were anesthetized on ice, decapitated, and extracted as described in Materials and Methods. ATP (a) and nitrite and nitrate (b) in the extracts were measured using luciferase-luciferin and an enhanced Griess reagent, respectively. Some flies were exposed to 22 ppm cyanide gas for 1 min prior to decapitation and extraction (cross-hatched bar, a). The data are the mean ± SD of at least three independent experiments performed in duplicate. In panel a, nitroprusside significantly decreased ATP compared to the control state (* $P < 0.05$), and cobinamide restored ATP to control values (pound symbol). In (b), nitroprusside significantly increased nitrite and nitrate levels compared to control values (* $P < 0.05$), and cobinamide had no effect on nitrite and nitrate levels in nitroprusside-treated flies (#).

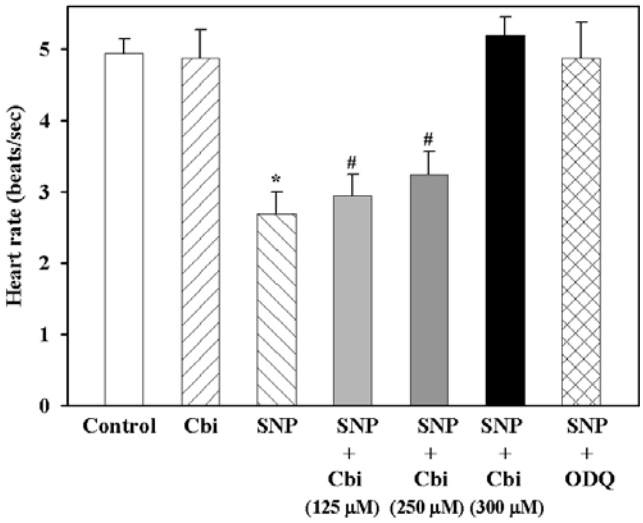


Figure 4. Effect of cobinamide on nitroprusside reduction of heart rate in *D. melanogaster*. Flies were injected with 1 μl of water (control, open bar), or 50 μ*M* nitroprusside (SNP) with or without the three indicated concentrations of cobinamide (Cbi). Some flies received 250 μ*M* cobinamide alone (left-diagonal bar) or the combination of 50 μM nitroprusside with 10 μ*M* ODQ (cross-hatched bar). Four hours later, the flies' heart rates were measured as described in Materials and Methods. The data are the mean ± standard error of the mean of at least three independent experiments performed on a minimum of 10 flies per condition. Nitroprusside significantly decreased heart rates compared to control flies (* $P < 0.05$), and adding cobinamide at 125 or 250 μ*M* was without effect (#).

release of NO and consequent stimulation of the enzyme guanylate cyclase, because the guanylate cyclase inhibitor ODQ returned heart rates and physical movement to control levels in nitroprusside-treated flies (Fig. 4 shows heart rates only). Although acutely exposing flies to cyanide gas reduces the flies' heart rates (data not shown), the lack of an apparent effect of cyanide in the present experiments may be due to the 4-hr time delay between injecting the flies with nitroprusside and measuring heart rates.

As we found previously (26), injecting flies with cobinamide had no effect on the flies' heart rate or physical activity: shown in Figure 4 is an injection of 1 μl of 250 μ*M* cobinamide, but similar results were found up to 500 μ*M* cobinamide. When cobinamide at concentrations 2.5–5 times that of nitroprusside was coinjected with nitroprusside, we observed small nonsignificant increases in heart rate and no changes in activity (Fig. 4; 125 and 250 μ*M* cobinamide were coinjected with 50 μ*M* nitroprusside). This is to be contrasted with 300 μ*M* cobinamide, which returned heart rates and activity fully to control levels in nitroprusside-treated flies (Fig. 4). Thus, up to a concentration five times that of nitroprusside, cobinamide appeared to bind only cyanide and had no effect on the NO-induced reduction in heart rate, but when increased to a concentration six times that of nitroprusside, cobinamide also bound NO and reversed the nitroprusside effects.

Cobinamide Detoxifies Nitroprusside-Released Cyanide in Mice Without Reducing the Hypotensive Effect of Nitroprusside. To study the effect of combining cobinamide with nitroprusside in a mammalian system, we treated mice with nitroprusside, and measured systolic and diastolic blood pressure, and serum and urinary thiocyanate concentrations (Fig. 5). We chose an intraperitoneal route of drug administration to have rapid absorption and ensure that the entire quantity of drug was given; we could not inject the drugs intravenously in the tail vein because the blood pressure measurements were being done on the tail. Measuring blood pressure repeatedly while delivering intraperitoneal medications required that the mice

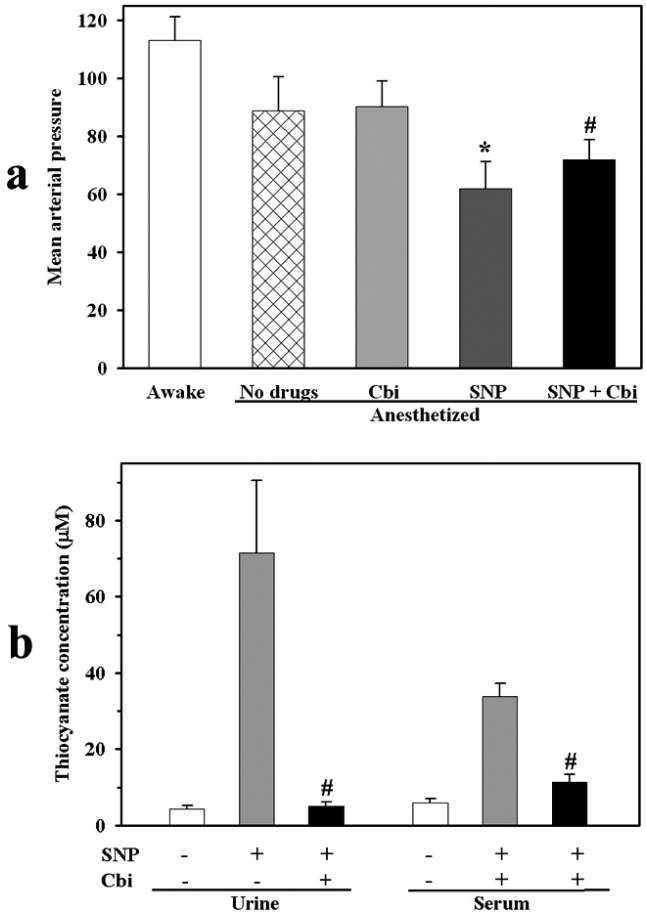


Figure 5. Effect of cobinamide on mean arterial blood pressure, and urine and serum thiocyanate concentrations in nitroprusside-treated mice. (a) Systolic and diastolic blood pressures were measured on the tail arteries of mice using a noninvasive automated instrument, with mean arterial pressures calculated by the instrument. Readings were taken while the mice were fully awake (open bar), after being anesthetized with 1.5% isoflurane (cross-hatched bar), and after being anesthetized and injected with 1 μ*M* cobinamide (Cbi) and/or 400 n*M* nitroprusside (SNP) (gray and black bars). The cobinamide was administered 30 mins before the nitroprusside. Each bar is the mean ± SD of 10 measurements performed over a 5-min interval on at least four mice. The effect of nitroprusside was significantly different from that of anesthetized mice not treated with drugs (* $P < 0.05$). Adding cobinamide to nitroprusside had no significant effect (#). (b) The mice described in the panel a experiments were allowed to recover from anesthesia and were euthanized 1 hr later; immediately thereafter, urine was obtained by bladder aspiration and blood was obtained by cardiac puncture. The samples were stored at 4°C, and thiocyanate was measured within 24 hrs. Open bars are from control mice not treated with nitroprusside (SNP) or cobinamide (Cbi), gray bars are from mice treated with nitroprusside alone, and black bars are from mice treated with nitroprusside and cobinamide. The data are the mean ± SD of three experiments performed in duplicate, with the reductions in urinary and serum thiocyanate by cobinamide in nitroprusside-treated mice being statistically significant (# $P < 0.01$).

were anesthetized, and we found that 1.5% isoflurane reduced the mean arterial pressure by an average of 24 mm Hg (Fig. 5a). Injecting the anesthetized mice with 400 nmol of nitroprusside reduced the mean arterial pressure by an additional 27 mm Hg (Fig. 5a). Cobinamide alone had no effect on blood pressure, and when 1 μmol was injected 30 mins prior to injecting nitroprusside, no significant effect on nitroprusside-induced hypotension was observed (Fig. 5a). Thus, at a concentration 2.5 times that of nitroprusside, cobinamide did not seem to bind NO.

Urinary thiocyanate was very high in nitroprusside-treated mice, and cobinamide reduced thiocyanate excretion to that of control non–nitroprusside-treated mice (Fig. 5b). Serum thiocyanate was also significantly reduced by cobinamide, although the effect was not as dramatic as observed in urine (Fig. 5b).

In addition to assessing urinary and serum thiocyanate concentrations as objective measures of cyanide scavenging by cobinamide, we also observed the mice for subjective effects of cyanide toxicity. We found that during recovery from anesthesia, mice that had been treated with nitroprusside behaved similarly to mice treated with sublethal doses of potassium cyanide (34). The mice were listless and had a broad-based gait with difficulties raising their bodies when walking; these abnormalities resolved slowly over about 4 hrs. Mice that did not receive nitroprusside, or those that had received nitroprusside plus cobinamide, recovered quickly from anesthesia, similar to control untreated mice.

Discussion

Nitroprusside is an effective drug for treating hypertensive emergencies and acute heart failure syndromes (3–5). Its major drawback is cyanide toxicity related to the release of five cyanide ions per NO molecule generated. A nontoxic agent that could neutralize the cyanide ions without interfering with the release of NO would allow nitroprusside to be administered more safely at higher doses and for longer periods. Cobinamide may be such an agent.

Compared with cobalamin (vitamin B_{12}), cobinamide lacks a dimethylbenzimidazole ribonucleotide group coordinated to the lower axial position of the cobalt atom (Fig. 6). The lack of the dimethylbenzimidazole group leads to three major differences between cobinamide and cobalamin. First, it provides cobinamide two ligand binding sites instead of only one, leading to a $K_{Aoverall}$ of cobinamide for cyanide of 10^{22} M^{-1} (35). Second, it increases the binding affinity of the upper ligand binding site, because the dimethylbenzimidazole group exerts a negative *trans* effect on that site; this is why the K_A for the binding of the first cyanide ion to cobinamide is 100 times greater than that for cobalamin (17, 35). Finally, it increases the solubility of cobinamide many-fold, making it potentially more useful as a pharmacological agent.[2]

Under ambient conditions, the cobalt atom in cobalamin and cobinamide is in the +3 valency state, designated as cobalamin(III) and cobinamide(III). Cyanide ion binds directly to cobinamide(III), but NO binds only to cobinamide with the cobalt in the +2 valency state (cobinamide[II]) (17). NO reduces cobinamide(III) to cobinamide(II),

[2] Balasubramanian M, Boss GR. Unpublished observations.

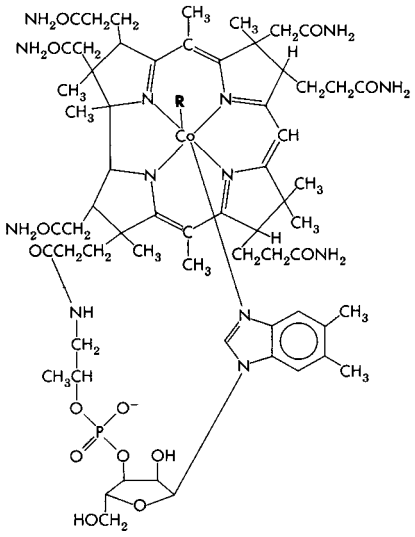


Figure 6. The chemical structures of cobalamin and cobinamide. The structure of cobalamin is shown. Cobinamide lacks the dimethylbenzimidazole ribonucleotide tail coordinated to the cobalt atom in the lower axial position. The "R" is an OH group in hydroxocobalamin and a cyanide ion in cyanocobalamin (vitamin B_{12}).

and in the process, the NO is oxidized to nitrite; a second NO can then bind to the cobinamide(II) (17). The rate-limiting step is cobinamide reduction, and it is not possible to calculate a K_A for this reaction. Thus, when we compared the relative affinities of cyanide and NO for cobinamide using the COMICS program, we actually overestimated NO's affinity because we used the affinity of NO for cobinamide(II). This may explain why the calculations indicated that less free NO would be available at a 5:1 molar ratio of cobinamide to nitroprusside than we observed experimentally in several different systems.

Cobinamide is present in human serum, bile, and tissues, probably because it is found in vitamin preparations (36–39). Its presence in vitamins likely occurs because vitamin B_{12} (cobalamin) is purified from bacteria, and as the penultimate precursor in cobalamin biosynthesis, cobinamide may copurify with cobalamin (38). Cobinamide is absorbed across hog ileum independently of intrinsic factor and, once absorbed, binds tightly to haptocorrin and poorly to transcobalamin II (40, 41). Cobinamide binding to haptocorrin should not change its affinity for cyanide, because we have shown that incubating cobinamide with human serum does not change its binding characteristics.[3]

Cobinamide had no effect on the growth of mouse leukemic cells, or human monocytes and lymphocytes, when used at low micromolar concentrations (42, 43). Similarly, when administered continuously for 14 days to rats at a dose of 2 μg/hr, cobinamide had no apparent toxic effects and did not inhibit methionine synthase or methylmalonyl-CoA mutase, the two mammalian cobalamin-dependent enzymes (44). We also found no inhibition of the latter two enzymes by cobinamide, but we did find that the drug inhibited growth of human and rodent fibroblasts at concentrations above 50 μ*M* (20). The growth inhibition was prevented completely by providing equimolar concentrations of cobalamin, suggesting that cobinamide may interfere with conversion of cobalamin to its biologically active forms (20).

Nitroprusside is generally used at doses of 0.25–10 μg/kg per min (2, 4). Using a midrange of 5 μg/kg per min, this would calculate to a serum concentration of 26.7 n*M*. Providing cobinamide up to a concentration five times that of nitroprusside would yield a serum cobinamide concentration of 133 n*M*, well below a concentration where toxic effects might occur.

The amount of nitroprusside we administered to the mice was about 5 μg/g. This is considerably more than the amount given to humans, but it is difficult to compare the mouse dose to the human dose because we administered the drug as a single intraperitoneal injection rather than as a continuous intravenous drip. However, even taking into consideration the time factor, the mouse dose was higher than that used in humans, and at these relatively high nitroprusside doses, cobinamide detoxified cyanide without interfering with nitroprusside's hypotensive effect.

Because cobinamide binds NO, and may have potential use in clinical states of excess NO such as septic shock (45), we were concerned that cobinamide's positive effects of detoxifying cyanide released by nitroprusside might be negated by its binding of NO. However, theoretical calculations and experiments in cultured cells, *D. melanogaster*, and mice all indicated that cobinamide could be used to detoxify cyanide without significantly affecting NO concentrations. Thus, the data suggest that cobinamide could be an effective adjunct to nitroprusside therapy in humans. Cobinamide could, therefore, make nitroprusside a more attractive agent in the treatment of hypertensive emergencies and other conditions in which the potent vasodilating properties of NO would be advantageous.

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[3] Sharma VS, Boss GR. Unpublished observations.

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