Cyanide Detoxification by the Cobalamin Precursor Cobinamide

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Cyanide is a highly toxic agent that inhibits mitochondrial cytochrome-*c* oxidase, thereby depleting cellular ATP. It contributes to smoke inhalation deaths in fires and could be used as a weapon of mass destruction. Cobalamin (vitamin B_{12}) binds cyanide with a relatively high affinity and is used in Europe to treat smoke inhalation victims. Cobinamide, the penultimate compound in cobalamin biosynthesis, binds cyanide with about 10^{10} greater affinity than cobalamin, and we found it was several-fold more effective than cobalamin in (i) reversing cyanide inhibition of oxidative phosphorylation in mammalian cells; (ii) rescuing mammalian cells and *Drosophila melanogaster* from cyanide toxicity; and (iii) reducing cyanide inhibition of Drosophila Malpighian tubule secretion. Cobinamide could be delivered by oral ingestion, inhalation, or injection to Drosophila, and it was as effective when administered up to 5 mins post-cyanide exposure as when given pre-exposure. We conclude that cobinamide is an effective cyanide detoxifying agent that has potential use as a cyanide antidote, both in smoke inhalation victims and in persons exposed to cyanide used as a weapon of mass destruction. Exp Biol Med 231:641–651, 2006

Key words: cyanide; cobalamin; cobinamide; Chinese hamster fibroblasts; *Drosophila melanogaster*

Introduction

Cyanide is a potent toxin with the LD_{50} of potassium cyanide (KCN) for animals in the range of 2–8 mg/kg, and in humans, as little as 50 mg may be fatal (1). It has been used throughout history as a homicidal and suicidal agent and was used in chemical warfare in World War I, in Nazi concentration camps in World War II (referred to as Zyklon B), and was likely used during the Iran-Iraq War in the early 1980s (2). Cyanide has the potential to be used as a weapon of mass destruction, particularly in closed spaces, such as airports and train stations (3–5).

Cyanide gas is generated during the combustion of any material containing carbon and nitrogen, including cotton, plastics, silk, and wool, and, thus, cyanide gas is produced in residential and industrial fires (6). With the advent of more synthetic-based materials used in construction, cyanide gas may be responsible for as many deaths from smoke inhalation as carbon monoxide (6–9). Cyanide binds to metalloenzymes, and its primary intracellular target is considered to be cytochrome-*c* oxidase (i.e., complex IV of the mitochondrial electron transport chain) (10).

Several antidotes for cyanide intoxication exist, including sodium nitrite, sodium thiosulfate, and hydroxocobalamin (vitamin B_{12a}) (11). Sodium nitrite generates methhemoglobin (ferric hemoglobin), which has a high affinity for cyanide but can no longer bind oxygen and, thus, in smoke inhalation victims can exacerbate the reduction in oxygen-carrying capacity induced by carbon monoxide (12). Sodium thiosulfate acts as a sulfur donor for the enzyme rhodanese, which detoxifies cyanide by converting it to thiocyanate, but rhodanese is limited both in tissue distribution and amount. Cobalamin binds cyanide with a relatively high affinity ($K_A \approx 10^{12}\ M^{-1}$) (13), but still 4 to 5 g are required when treating victims of smoke inhalation. In the United States, sodium nitrite and sodium thiosulfate are used as cyanide antidotes, while in France and several other European countries, hydroxo-cobalamin is favored (14).

Cobinamide is the penultimate precursor in the biosynthesis of cobalamin, lacking the dimethylbenzimidazole nucleotide tail coordinated to the cobalt atom in the lower axial position (Fig. 1). Thus, whereas cobalamin has only an upper ligand binding site, cobinamide has both an upper and a lower ligand binding site; moreover, the dimethylbenzimidazole group has a negative trans effect on the upper binding site, thereby reducing cobalamin's affinity for ligands (15). The net effect is that cobinamide has a much greater affinity for cyanide ion than cobalamin, with a K_A of

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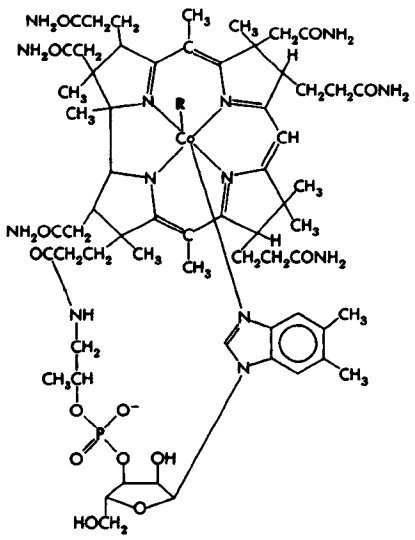

Figure 1. Structures of cobinamide and cobalamin. Shown is the structure of cobalamin; cobinamide lacks the dimethylbenzimidazole nucleotide tail linked to the cobalt atom in the lower axial position. The "R" is a cyanide group in cyanocobalamin (vitamin B_{12}).

$\approx 10^{22}$ M^{-1} (13), indicating that cobinamide should be a more effective cyanide detoxifying agent than cobalamin. However, one cannot assume that *in vitro* affinities will translate to *in vivo* conditions; for example, intracellular proteins may bind cobinamide and cobalamin to varying degrees, and it was, therefore, important to compare the cyanide detoxifying properties of cobinamide and cobalamin under physiologic conditions. We now show both in cultured mammalian cells and an intact organism that cobinamide is superior to cobalamin as a cyanide detoxifying agent.

Materials and Methods

Materials. Cobinamide was produced by acid hydrolysis of cobalamin (Sigma Chemical Co., St. Louis, MO), as described previously (16); unless noted otherwise, "cobinamide" and "cobalamin" refer to their hydroxo derivatives. The purity of cobinamide was assessed spectrophotometrically and by elution as a single peak by high-performance liquid chromatography on a reversed-phase column, with the eluent monitored by UV absorption at 348 nm (16). Potassium cyanide (Fisher Scientific, Pittsburgh, PA) was dissolved in 10 m*M* NaOH, and experiments, where KCN was injected into flies, it was diluted into 10 m*M* Na_2CO_3, pH 9.5. Chinese hamster lung fibroblasts (Chinese hamster cells) obtained from the American Type Culture Collection (CCL 16, Manassas, VA) were grown as described previously (17). Wild-type Oregon R *Drosophila melanogaster* from the Bloomington Stock Center (Bloomington, IN) were used as described previously (16). Beveled 33-gauge needles and 2.5-μl syringes were from the Hamilton Company (Reno, NV).

Measurement of Respiratory Activity of Chinese Hamster Cells. Mitochondrial respiratory activity of Chinese hamster cells was assessed by measuring oxygen consumption as described previously (17). Briefly, cells were harvested by trypsinization, resuspended in 20 m*M* Hepes (pH 7.1), 250 m*M* sucrose, and 10 m*M* $MgCl_2$, and were permeabilized with 100 mg/ml digitonin. An amount of permeabilized cells corresponding to ≈ 0.5 mg of protein was transferred to a metabolic chamber maintained at 37°C. The chamber was filled completely with the Hepes-sucrose-$MgCl_2$ buffer; we were careful to assure that no air bubbles were present. Cellular oxygen consumption was measured polarographically with a Clark oxygen electrode under basal conditions after stimulation by 5 m*M* sodium succinate and glycerol 3-phosphate and after adding 250 μ*M* KCN, followed by variable amounts of cobinamide or cobalamin.

Measurement of Growth of Cyanide-Treated Chinese Hamster Cells. Cells were grown in glucose-free Dulbecco's modified Eagle's medium supplemented with 25 m*M* galactose and 10% fetal bovine serum, as described previously (17). After 24 hrs of equilibration in the medium, 100 μ*M* KCN was added (referred to as zero time); KCN was additionally added at 8 and 24 hrs, and cells were counted at 48 hrs using a Model ZM Coulter Counter. To some cultures, 10 μ*M* cobinamide was added at zero time.

Delivery of Cobinamide and Cobalamin to *D. melanogaster*. *Ingestion of Cobinamide and Cobalamin.* Flies were grown on food containing cobinamide or cobalamin, as described previously (16). Briefly, standard fly food paste was liquified by heating to 40°C, and after adding cobinamide or cobalamin to final concentrations of 100 μ*M*, the food was cooled to room temperature. Flies were grown on the cobinamide- or cobalamin-supplemented medium from the first instar larval stage prior to use in experiments. We observed no toxicity from either agent, even when flies were grown for more than 10 generations on the supplemented food.

Injection of Cobinamide and Cobalamin. Flies anesthetized on ice were injected into the thorax with 1 μl of water, or with 1 μl of 500 μ*M* cobinamide or cobalamin dissolved in water, using a 33-gauge needle attached to a 2.5-μl syringe. They were allowed to recover for 10 mins and were then exposed to HCN, as described below. In some experiments, they were exposed to HCN first and were injected within 1 min of HCN exposure.

Inhalation of Cobinamide and Cobalamin. The mouthpiece of a nebulizer (EasyMist, Prestige Medical, Northridge, CA) was attached to a 1 × 3-cm chamber consisting of plastic tubing; gauze with cotton wool at the proximal end of the chamber reduced the rate of air flow and minimized turbulence. After a 10-min equilibration in the chamber, flies were exposed for 2 mins to nebulized water or nebulized 100 μ*M* cobinamide or 100 μ*M* cobalamin. The flies were allowed to recover for 2 mins prior to further treatment.

Exposure of *D. melanogaster* to HCN and KCN. *Exposure to HCN.* Flies were transferred to a 10-

ml plastic vial, and after a 10-min equilibration period, HCN was generated in the vial by spotting 1 μl of a 1 or 10 m*M* KCN solution on a 0.5 × 0.5-cm square of Whatman #1 filter paper, which was placed immediately in the vial. The vial was shaken gently for 20 secs to force the insects to fly and open their respiratory spiricles, and after a 1-min total HCN exposure, the paper square was removed. The HCN caused all flies, including those previously treated with cobinamide or cobalamin, to fall motionless to the bottom of the vial. Flies were monitored for activity, and those able to walk or fly within 1 hr were considered recovered. We showed in control experiments that water spotted on the Whatman paper had no effect on the flies and that pretreating the paper with 10 m*M* NaOH completely prevented subsequent toxicity of KCN, indicating that the paper was sufficiently acidic to generate HCN. Consistent with the latter point, we showed that by leaving the filter paper in the vial for 1 hr (without flies), we could quantitatively recover KCN spotted on the paper in NaOH in the bottom of the vial (as described below). Since not all of the KCN was necessarily released as HCN during the 1-min exposure of the flies, and since some of the generated HCN gas could condense into liquid at room temperature, the stated concentrations of HCN gas represent the maximal concentration to which the flies were exposed.

Injection with KCN. Anesthetized flies were injected with 1 μl of 10 m*M* Na_2CO_3, pH 9.5, or 1 μl 100 μ*M* KCN dissolved in the Na_2CO_3 solution, as described above for injection with cobinamide or cobalamin.

Measurement of HCN. Cyanide gas generated in vials was measured by collecting the HCN in 0.2 ml of 100 m*M* NaOH in the bottom of the vials, being careful not to allow the paper containing the KCN to contact the NaOH. Cyanide in flies exposed to HCN was measured by extracting 20 flies in 500 μl of 100 m*M* NaOH. In both cases, the resulting NaCN was measured by incubation with p-nitrobenzaldehyde and o-dinitrobenzene, with the colored product measured at 578 nm (18). The assay was linear between 1 and 15 μ*M* NaCN.

Effect of KCN on Malpighian Tubule Secretion in *D. melanogaster*. The Malpighian tubules of *D. melanogaster* are the insect's fluid transporting and osmoregulatory organ, corresponding to vertebrate kidneys. We measured rates of tubular secretion as described previously (16). Briefly, the two pairs of Malpighian tubules of a fly were dissected from 10 adult flies, which had been grown on either standard food or food containing 100 μ*M* cobinamide or cobalamin. Each tubule pair with its accompanying ureter was suspended in mineral oil, with the nonureteral end of one tubule bathed in a 10-μl droplet of Schneider's Insect medium and the corresponding end of the other tubule immobilized on a dissecting pin. The amount of fluid transported by the tubule bathed in Schneider's medium was determined every 10 mins at room temperature by measuring the size of drops formed at the end of the ureter. After measuring basal fluid secretion rates over three 10-min intervals, KCN was added at a final concentration of 100 μ*M* to the droplet of Schneider's medium, and rates of fluid secretion were measured for three additional 10-min intervals.

Statistical Analyses. Differences between sets of data were compared using a non-paired *t* test.

Results

Cobinamide Recovers Respiratory Activity in Cyanide-Treated Chinese Hamster Cells. We have shown previously that measuring respiratory activity in permeabilized Chinese hamster cells incubated with succinate and glycerol 3-phosphate accurately reflects cytochrome-*c* oxidase activity (17). Cyanide is an effective inhibitor of cytochrome-*c* oxidase, and we found that 250 μ*M* KCN almost completely inhibited respiratory activity in Chinese hamster cells (Fig. 2a). At physiologic pH, KCN will be converted to HCN, since the pKa of the latter is 9.3; although the boiling point of HCN is 25.7°C and the experiments were performed at 37°C, it is unlikely that any of the generated HCN escaped from the buffer, since the incubation chamber was filled completely with buffer and sealed tightly. Adding cobinamide rapidly increased respiratory activity, and at an equimolar concentration as KCN, full recovery occurred (Fig. 2a, upper tracing shows cobinamide at 150 μ*M*, and Fig. 2b, closed circles, shows a dose-response from 50 to 300 μ*M* cobinamide). Cobalamin was less potent than cobinamide, inducing a maximum 42% recovery of respiration at 250–300 μ*M* (Fig. 2a, lower tracing shows 250 μ*M* cobalamin, and Fig. 2b, open circles shows a dose-response). Neither cobinamide nor cobalamin alone had any effect on oxygen consumption.

Cobinamide Restores Growth in Cyanide-Treated Chinese Hamster Cells. If glucose is available, cultured mammalian cells can derive all of their ATP from glycolysis when oxidative phosphorylation is inhibited (19). Thus, to study cyanide's effect on cell growth, we had to use glucose-free medium containing galactose as a sugar source (17). Because KCN will be converted to HCN under physiologic conditions, and because HCN is volatile, we had to add KCN serially over time. Treating Chinese hamster cells with 100 μ*M* KCN at zero time and at 8 and 24 hrs inhibited growth at 48 hrs by 31% ± 5% (mean ± standard deviation [SD] of three independent experiments). Adding 10 μ*M* cobinamide at zero time restored the cell's growth rate to normal. Presumably the reason low amounts of cobinamide were effective is that most of the generated HCN vaporized from the culture medium.

Cobinamide Detoxifies Cyanide in *D. melanogaster*. *Delivery of Cobinamide via Ingestion.* *Drosophila melanogaster* is recognized to be a model for human disease and is used increasingly in drug discovery (20). As a flying insect, *D. melanogaster* has a high metabolic rate and would be expected to be highly sensitive to HCN. The gas could enter flies within seconds through their spiricle-

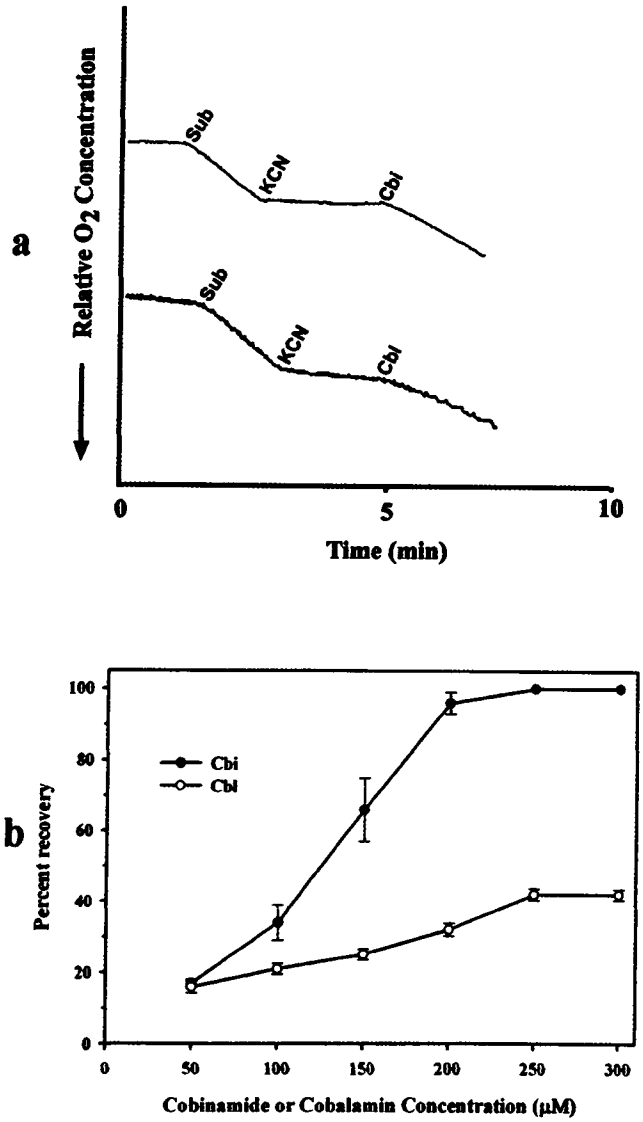

Figure 2. Cobinamide recovers respiratory activity in cyanide-treated Chinese hamster cells. Chinese hamster cells at a density of $\approx 3 \times 10^7$ cells/ml were transferred to a metabolic chamber, and respiratory activity was assessed by measuring oxygen consumption using an oxygen electrode. In the absence of added substrate (sub, 5 m*M* sodium succinate and 5 m*M* glycerol 3-phosphate), oxygen consumption was low—as indicated by the almost horizontal lines on the tracings in panel a. KCN was added to a final concentration of 250 μ*M* in all experiments, and cobinamide (Cbi) and cobalamin (Cbl) were added at 150 and 250 μ*M*, respectively, in panel a, and at the indicated concentrations in panel b. Panel a shows raw data from two experiments plotted on the same graph, and panel b shows percent recovery by cobinamide and cobalamin; the latter data are a summary of three experiments performed in duplicate (mean ± SD).

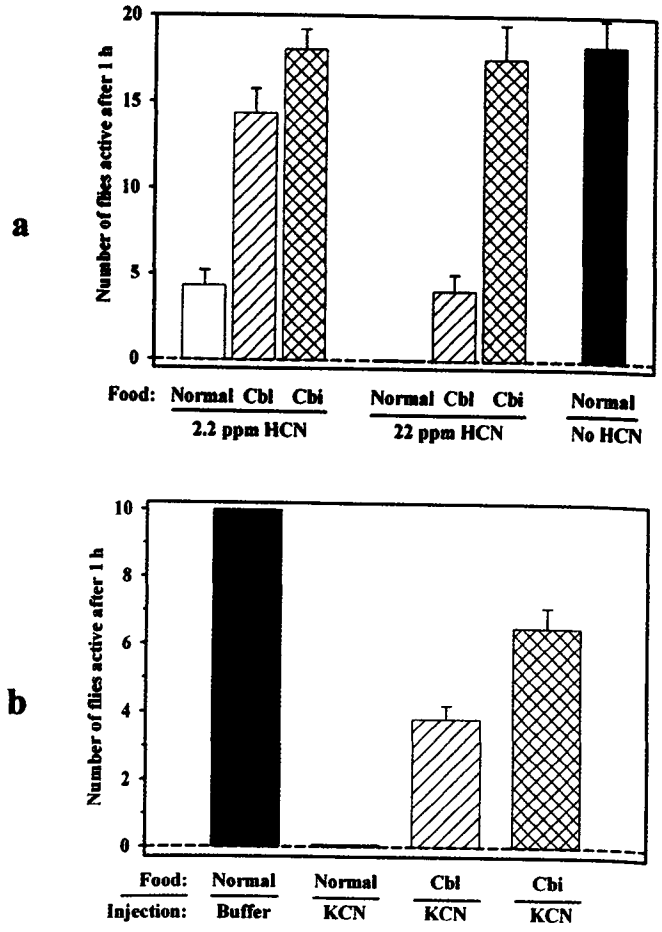

Figure 3. Cobinamide ingestion detoxifies cyanide gas and KCN in *D. melanogaster.* Flies were grown from the first instar larval stage on standard fly food paste (normal food, open and filled bars) or standard food containing either 100 μ*M* cobalamin (Cbl, left-diagonal striped bars) or 100 μ*M* cobinamide (Cbi, cross-hatched bars). (a) Under each indicated condition, 20 flies were exposed for 1 min to HCN at either 2.2 ppm or 22 ppm. The HCN caused all flies to collapse motionless within 20 secs; flies not exposed to HCN are shown in the filled bar on far right. (b) Under each condition, 10 flies were injected with 1 μl of either 10 m*M* Na_2CO_3, pH 9.5 (filled bar), or 100 μ*M* KCN in Na_2CO_3, as indicated. In both panels, the data plotted on the ordinate are the number of flies that recovered and were able to walk or fly by 1 hr postexposure and represent the mean ± SD of at least three independent experiments performed in duplicate.

tracheal respiratory system and more slowly over minutes through transcuticle absorption (21). We found that a 1-min exposure to an HCN concentration as low as 2.2 ppm killed 80% of the flies, and at 22 ppm, all flies died (Fig. 3a, open bar, and solid line at zero value on *y* axis, respectively, for flies grown on normal food). At the low and high exposure levels, we found the HCN concentration in the flies was 1 and 10 μ*M*, respectively, based on a fluid volume of about 10 μl in the flies (21); human fatalities occur at cyanide concentrations above 40 μ*M* (1). When flies were grown on food containing 100 μ*M* cobinamide and then exposed to HCN, 90% survived the gas exposure, both at 2.2 and 22 ppm (Fig. 3a, cross-hatched bars). Survival of the cobinamide-fed flies was similar to that of mock-treated flies not exposed to HCN (Fig. 3a, filled bar). This is to be contrasted with flies fed 100 μ*M* cobalamin, which exhibited 80% recovery at 2.2 ppm HCN, but only 20% recovery at 22 ppm (Fig. 3a, left-diagonal striped bars; $P < 0.01$ for comparison between cobinamide and cobalamin at 22 ppm). The cobinamide and cobalamin concentrations in the flies could not be determined because they were below

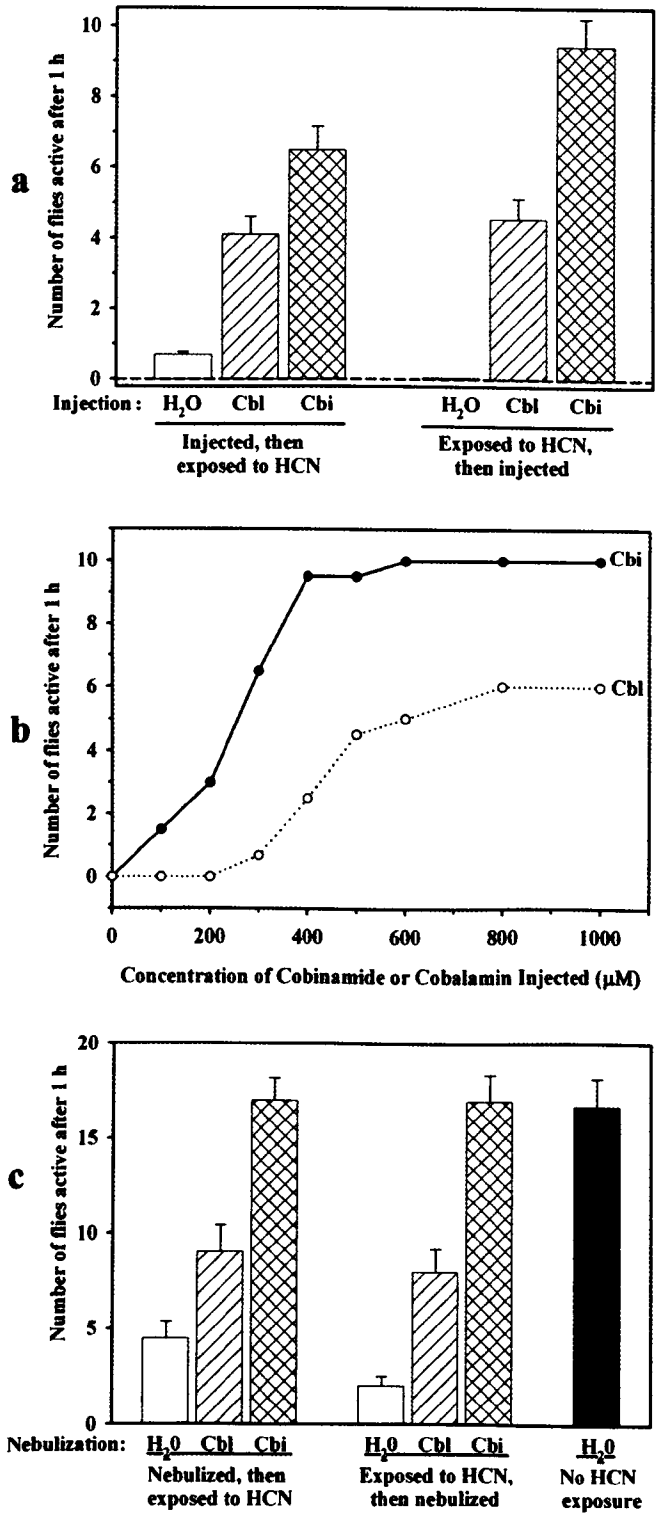

Figure 4. Cobinamide injection or inhalation detoxifies cyanide gas in *D. melanogaster*. (a, c) Flies received water (open and filled bars), cobalamin (Cbl, left-diagonal striped bars), or cobinamide (Cbi, cross-hatched bars) by either injection (1 μl of water or 500 μ*M* Cbl or Cbi, panel a) or inhalation using a nebulizer containing water or 100 μ*M* of Cbl or Cbi (panel c). (b) Flies were injected with 1 μl of the indicated concentrations of either cobinamide (Cbi) or cobalamin (Cbl). The flies were exposed for 1 min to HCN at 22 ppm (panels a and b) or 2.2 ppm (panel c), either after receiving the cobinamide or cobalamin (bars in left half of panels a and c) or before receiving the cobinamide or cobalamin (bars in right half of panels a and c, and panel b). Flies nebulized with water, but not exposed to HCN, are shown in the filled bar in panel c. In panels a and c, the data are the mean ± SD of at least three independent experiments performed in duplicate on 10 and 20 flies, respectively; in panel b, each data point is the mean of at least two independent experiments performed on 10 flies.

the limits of detection of the high-performance liquid chromatography system described in the Materials and Methods section (100 pmol), even when extracts from 20 flies were combined.

The cobinamide-fed flies could be exposed to 22 ppm HCN for up to 15 mins without any significant increase in mortality, but exposure times >30 mins killed all flies. This indicates that in addition to entering through the respiratory system, HCN may, during more prolonged exposure, enter the flies through other means (e.g., a transcuticle mechanism, thereby achieving higher tissue levels).

As an alternative method of exposing flies to cyanide, we injected them with 1 μl of 100 μ*M* KCN dissolved in 10 m*M* Na_2CO_3, pH 9.5. Assuming the KCN was evenly distributed in the flies, the intracellular KCN concentration should have been about 10 μ*M*, similar to the concentration in flies exposed to 22 ppm HCN. Injecting Na_2CO_3 alone had no effect on the flies (Fig. 3b, solid bar), whereas injecting KCN resulted in 100% mortality (Fig. 3b, solid line at zero value on *y* axis). Flies grown on cobinamide were relatively resistant to the KCN, exhibiting a 65% survival rate (Fig. 3b, cross-hatched bar), while flies grown on cobalamin exhibited a 38% survival rate (Fig. 3b, left diagonal bar; $P < 0.01$ for difference between cobinamide and cobalamin).

Delivery of Cobinamide via Injection. Because we could not measure the concentration of cobinamide and cobalamin achieved in the flies in the ingestion studies, we injected the drugs, which allowed us to know the exact amount received by the flies. In the first series of experiments, we injected flies with 1 μl of 500 μ*M* cobinamide or cobalamin, yielding approximate concentrations of 50 μ*M* in the flies, and we then exposed the flies for 1 min to 22 ppm HCN. This exposure to HCN killed almost all flies injected with water (Fig. 4a, open bar), similar to our previous findings. This is to be contrasted with flies injected with cobinamide or cobalamin, of which 65% and 41% survived, respectively (Fig. 4a, cross-hatched bar and left diagonal bar on left, respectively; $P < 0.05$ for difference between cobinamide and cobalamin). The combination of cobinamide and cobalamin was no more effective than cobinamide alone.

In treating patients exposed to HCN, it would be most useful to have an agent that could be used postexposure, rather than pre-exposure, and we therefore modified the protocol and exposed the flies to HCN for 1 min, followed by injecting them with water, cobinamide, or cobalamin. We found that 500 μ*M* cobinamide and cobalamin salvaged

94% and 45% of flies exposed to HCN, respectively, whereas water-injected flies all died (Fig. 4a, cross-hatched bar and left diagonal bar on right, respectively, compared to solid line at zero value on *y* axis; $P < 0.01$ for difference between cobinamide and cobalamin). The reason for higher survival rates when cobinamide was administered post–HCN exposure, compared to pre–HCN exposure, may reflect a procedural difference: in the pre–HCN exposure experiments, the flies were anesthetized on ice to allow injection, which could have decreased their subsequent survival, whereas in the post–HCN exposure experiments, the flies were injected immediately after HCN exposure, since they were already sedated. As part of these studies, we found that cobinamide could be given up to 5 mins after HCN exposure and still rescue the flies as effectively as when it was given immediately after cyanide.

These injection experiments allowed us to compare potencies of cobinamide and cobalamin; cobinamide rescued 100% of flies at 600 μM, whereas cobalamin concentrations as high as 1 mM rescued only 60% of flies (Fig. 4b). Drug concentrations required to rescue half of the flies were 250 μM for cobinamide, and 600 μM for cobalamin. Thus, cobinamide was at least 2.4 times more potent than cobalamin and could rescue all flies exposed to HCN, compared to cobalamin, which rescued less than two-thirds of the flies. These data are similar to those found when comparing the potencies of cobinamide and cobalamin in reversing cyanide inhibition of oxidative phosphorylation (Fig. 2b).

Delivery of Cobinamide via Inhalation. In acute cyanide gas exposure, administering cobinamide *via* the gastrointestinal route would unlikely result in sufficiently rapid absorption to be effective, and administering cobinamide *via* injection would require trained health personnel. We therefore tested whether cobinamide could reduce cyanide toxicity when provided *via* inhalation, since this mode of delivery is simple and rapid. We developed a system using a hand-held nebulizer to deliver drugs to flies *via* inhalation, and we showed, using the red dye amaranth, that the dye was delivered to internal organs *via* the flies' spiricle-tracheal system. To our knowledge, this is the first example of administering drugs to *D. melanogaster via* nebulization. Even at low flow rates, nebulization of water killed about 20% of control, non–cyanide exposed flies, presumably because of turbulent air flow and shear forces (Fig. 4c, filled bar). Exposing flies to 2.2 ppm HCN after nebulization of water killed 80% of the flies (Fig. 4c, open bar on left), a result similar to mortality rates found previously at this HCN concentration (Fig. 3a). However, only 18% of flies died that had received cobinamide by nebulization prior to HCN exposure (Fig. 4c, cross-hatched bar on left). Since the latter survival rate is similar to that of non–cyanide exposed flies receiving nebulized water, cobinamide completely prevented mortality. Flies that received cobalamin by nebulization exhibited some protection from HCN, with a 55% mortality rate (Fig. 4c, left diagonal bar on left; $P < 0.01$ for difference between cobinamide and cobalamin).

As mentioned above, cobinamide would be most useful in cases of cyanide exposure if it could be administered postexposure, rather than pre-exposure. We therefore altered the protocol and exposed flies to 2.2 ppm HCN and then subjected them to nebulization with water, cobinamide, or cobalamin. We found cobinamide was as effective in preventing death under these conditions as when it was administered prior to cyanide exposure (Fig. 4c, cross-hatched bar on right). Cobalamin also exhibited similar results when administered prior to cyanide exposure, but flies nebulized with water showed lower survival rates, presumably because they were already impaired from the cyanide at the time of nebulization (Fig. 4c, open and left-diagonal bars on right, respectively).

Cobinamide Reduces Cyanide Inhibition of Malpighian Tubule Secretion. Secretion by insect Malpighian tubules is an ATP-dependent process and would be expected to be inhibited by cyanide (22). We found that 100 μM KCN rapidly reduced rates of tubular secretion by *D. melanogaster* Malpighian tubules (Fig. 5a, filled circles). Tubules that had been treated at zero time with either 100 μM cobinamide or 100 μM cobalamin showed significant resistance to the inhibitory effect of KCN (Fig. 5a, open circles and triangles, respectively). Cobinamide and cobalamin had no effect on basal secretion rates, as can be observed during the zero to 30-min interval. When the same experiment was performed on flies that had been grown on cobinamide prior to measuring tubular secretion rates, we found almost complete reversal of the toxic effects of KCN (Fig. 5b, compare open circles, cobinamide-fed flies to filled circles, control flies). The more complete reversal of KCN toxicity in the cobinamide-fed flies compared to the cobinamide-treated tubules may have resulted from slow cellular uptake of cobinamide in the latter condition; longer times of cobinamide preincubation with the tubules could not be performed because the tubules have a limited experimental time. Flies that had been grown on cobalamin showed an intermediate degree of resistance to KCN (Fig. 5b, triangles).

To determine if cobinamide could reverse the inhibitory effects of cyanide on tubular secretion, we added cobinamide 10 mins after adding 100 μM KCN to the tubules and found that tubular secretion rates increased significantly (Fig. 5c, open circles are cobinamide-treated tubules). Even after only 10 mins of cyanide exposure, we observed morphologic changes in the tubular cells, which could explain why cobinamide did not return tubular secretion to normal.

Discussion

Drosophila is emerging as a model organism in drug discovery for many reasons, including its rapid rate of propagation, its well-defined genetics, and its similarity to

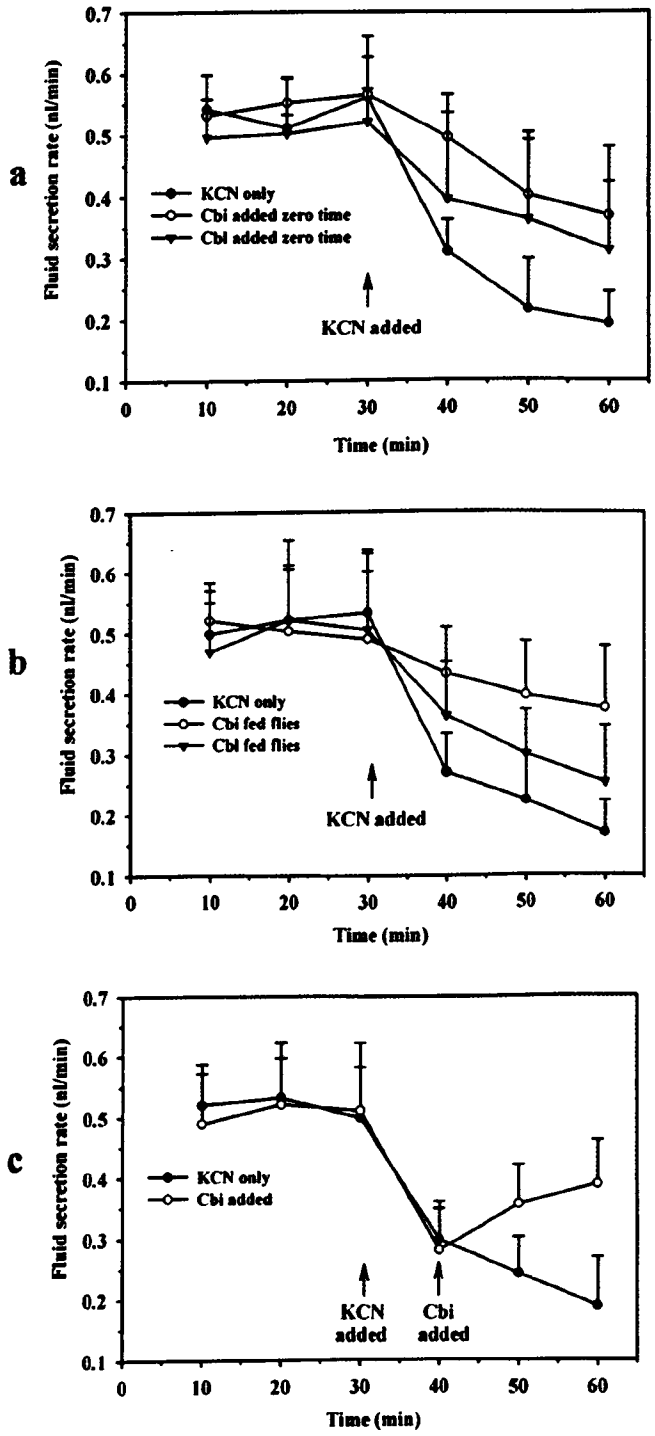

Figure 5. Cobinamide reduces cyanide inhibition of Malpighian tubule secretion. Pairs of Malpighian tubules were removed from *D. melanogaster*, and rates of fluid secretion were determined by measuring droplet formation from the ureter over 10-min intervals. Secretion rates were measured for three 10-min intervals prior to adding 100 μM KCN to all tubules. (a) Cobinamide (open circles) and cobalamin (triangles) at concentrations of 100 μM were added to the tubules at zero time. (b) Flies were grown from the first instar larval stage on standard fly food (closed circles) or food containing either 100 μM cobinamide (open circles) or 100 μM cobalamin (triangles) prior to removal of their Malpighian tubules. (c) Cobinamide at a concentration of 100 μM was added to the tubules 10 mins after adding KCN (open circles). In all three panels, data are the mean ± SD of at least three independent experiments performed on 20 pairs of Malpighian tubules.

mammalian systems (20, 23, 24). As we show in this study, drugs can be administered to Drosophila *via* multiple mechanisms, further enhancing its use in pharmacologic research. Use of Drosophila in drug discovery will not eliminate the need for vertebrate studies, but it should reduce the number of animals required.

Cobalamin has been used both prophylactically and therapeutically to treat experimental cyanide toxicity, and when given at high enough doses, it has rescued mice and guinea pigs from cyanide-induced apnea and coma (25, 26). Cobalamin has been used for some time in France to treat human cyanide exposure, either alone or combined with sodium thiosulfate, and several well-documented cases involving revival of unresponsive comatose patients have been published (27, 28). The major disadvantage of cobalamin is that high doses are required, generally 4–5 g must be administered intravenously. We found that cobinamide detoxified cyanide more effectively than cobalamin in two separate biological systems, and although it is difficult to strictly compare the two agents, because even at high doses cobalamin never achieved the efficacy of cobinamide, clearly cobinamide is at least two to three times more potent than cobalamin. Taking into account the lower molecular weight of cobinamide compared to cobalamin, the data indicate that 1–1.5 g of cobinamide should be sufficient to treat severe cyanide toxicity in humans. Although this is still a relatively high dose, it is well within a clinical range.

Previous workers have shown that HCN, the form of cyanide present at physiologic pH, reacts with purified cytochrome-*c* oxidase in two steps: relatively rapid formation of an enzyme-HCN intermediate, followed by slow conversion of the intermediate to a stable product, possibly an enzyme-cyanide ion complex (29, 30). The rate constant for the first step is 0.03 sec^{-1}, yielding a half-life of the intermediate of 23 secs (29, 30). Dissociation of cyanide from the final product is slow, 10^6 sec^{-1}, and thus the overall reaction of cyanide with cytochrome-*c* oxidase has been referred to as irreversible or "quasi-reversible" (29, 30). Using cobinamide and cobalamin, both of which have relatively high affinities for cyanide, we found that the reaction of cyanide with cytochrome-*c* oxidase was reversible, as demonstrated by measuring enzyme activity in permeabilized Chinese hamster cells. In addition, cobinamide and cobalamin reversed the lethal effects of cyanide in the hamster cells and in *D. melanogaster*, and cobinamide largely reversed cyanide inhibition of Malpighian tubular transport, an ATP-dependent process. Assuming these latter effects of cyanide were from inhibition of cytochrome-*c* oxidase, these data provide evidence that the reaction of cyanide with cytochrome-*c* oxidase is reversible when assessed in intact organisms or organ systems. Similar conclusions have been drawn from previous workers using cobalamin in other animal models of cyanide poisoning and in treating smoke inhalation victims (14, 25–27).

When developing responses to weapons of mass

destruction, it would be helpful to have drugs that could be administered after exposure to the toxic agent, since prophylactic treatment may not be possible. It was therefore useful to find that cobinamide could be given to flies after cyanide exposure and that it was at least as effective as when given prior to cyanide exposure. Of perhaps greater importance, cobinamide could be administered by injection or inhalation, which would allow for rapid drug delivery.

Recent data indicate that cyanide may play a role in the development of several diseases. Cigarette smokers have high levels of blood and urinary cyanide and thiocyanate, as do patients on hemodialysis (31, 32). The high thiocyanate levels may contribute to lipid oxidation and, therefore, arteriosclerosis, because thiocyanate is an excellent substrate for peroxidase enzymes (33–36). Some *Pseudomonas aeruginosa* strains are cyanogenic, including those isolated from sputa of cystic fibrosis patients, indicating that cyanide may contribute to the lung destruction in cystic fibrosis patients infected with *P. aeruginosa* (37). For these clinical conditions it could be useful to have a drug that could detoxify cyanide, particularly one that could be administered by inhalation in the case of patients with cystic fibrosis.

As a precursor in the biosynthesis of cobalamin, cobinamide is a contaminant of multivitamin preparations, and at least in pigs, it can be absorbed across the ileum independently of intrinsic factor (38, 39). Once absorbed, cobinamide binds tightly to haptocorrin but poorly to transcobalamin II (40–42). Since the serum levels of haptocorrin and transcobalamin II are similar, a significant amount of cobinamide could be present in serum, and cobalamin analogs, including cobinamide, have been detected in the serum, bile, and tissues of animals and humans, accounting for 5%–50% of total corrinoids (41, 43–46). Intravenous injection of radioactive cobinamide into rabbits indicates that cobinamide, like cobalamin, is taken up by the liver within minutes, but unlike cobalamin, cobinamide is released more slowly from the liver; once released from the liver, the tissue distribution and urinary and fecal excretion of the two agents is similar (47). Thus, with a few notable exceptions, cobinamide and cobalamin appear to be handled similarly in mammalian systems.

At concentrations up to 200 μM, cobinamide did not inhibit growth of mouse leukemic cells or human monocytes and lymphocytes (48, 49). It had no effect on the growth of baby chicks when administered parenterally at 40 times the dose of cobalamin, but it did inhibit chick growth when given orally, which indicates that it interfered with cobalamin absorption (50). Cobinamide was nontoxic to rats when administered continuously at 4 μg/hr for 14 days, and it did not inhibit the two mammalian cobalamin-dependent enzymes methionine synthase and methylmalonyl-CoA mutase (51). We found that up to 50 μM, cobinamide was not toxic to cultured mammalian cells and that toxicity at higher concentrations could be reversed completely by cobalamin (16). We also found no effect of cobinamide on the activities of methionine synthase or methylmalonyl-CoA mutase, indicating that cobinamide was interfering with cobalamin metabolism (16). Since we found that cobinamide and cobalamin yielded similar results as cobinamide alone, the two corrinoids could be used together to avoid cobinamide toxicity.

In conclusion, cobinamide is an effective cyanide detoxifying agent that could be used potentially in a variety of clinical states, and it might be particularly beneficial as an antidote to massive cyanide poisoning.

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1. Salkowski AA, Penney DG. Cyanide poisoning in animals and humans: a review. Vet Hum Toxicol 36:455–466, 1994.
2. Way JL. Cyanide intoxication and its mechanism of antagonism. Annu Rev Pharmacol Toxicol 24:451–481, 1984.
3. Greenfield RA, Brown BR, Hutchins JB, Iandolo JJ, Jackson R, Slater LN, Bronze MS. Microbiological, biological, and chemical weapons of warfare and terrorism. Am J Med Sci 323:326–340, 2002.
4. Rotenberg JS. Cyanide as a weapon of terror. Pediatr Ann 32:236–240, 2003.
5. Eckstein M. Cyanide as a chemical terrorism weapon. JEMS 29:22–31, 2004.
6. Alcorta R. Smoke inhalation & acute cyanide poisoning. Hydrogen cyanide poisoning proves increasingly common in smoke-inhalation victims. JEMS 29:6–15, 2004.
7. Alarie Y. Toxicity of fire smoke. Crit Rev Toxicol 32:259–289, 2002.
8. Esposito FM, Alarie Y. Inhalation toxicity of carbon monoxide and hydrogen cyanide gases released during the thermal decomposition of polymers. J Fire Sci 6:195–242, 1988.
9. Silverman SH, Purdue GF, Hunt JL, Bost RO. Cyanide toxicity in burned patients. J Trauma 28:171–176, 1988.
10. Scheffler IE. Mitochondria make a come back. Adv Drug Delivery Rev 49:3–26, 2001.
11. Gracia R, Shepherd G. Cyanide poisoning and its treatment. Pharmacotherapy 24:1358–1365, 2004.
12. Moore SJ, Norris JC, Walsh DA, Hume AS. Antidotal use of methemoglobin forming cyanide antagonists in concurrent carbon monoxide/cyanide intoxication. J Pharmacol Exp Ther 242:70–73, 1987.
13. Hayward GC, Hill HAO, Pratt JM, Vanston NJ, Williams ARW. The chemistry of vitamin B(12). Part IV. 1. The thermodynamic trans-effect. J Chem Soc 6485–6493, 1965.
14. Fortin JL, Ruttiman M, Domanski L, Kowalski JJ. Hydroxocobalamin: treatment for smoke inhalation-associated cyanide poisoning. Meeting the needs of fire victims. JEMS 29:18–21, 2004.
15. Sharma VS, Pilz RB, Boss GB, Magde D. Reactions of nitric oxide with vitamin B12 and its precursor, cobinamide. Biochemistry 42: 8900–8908, 2003.
16. Broderick KE, Singh V, Zhuang S, Kambo A, Chen JC, Sharma VS, Pilz RB, Boss GR. Nitric oxide scavenging by the cobalamin precursor cobinamide. J Biol Chem 280:8678–8685, 2005.
17. Soderberg K, Mascarello JT, Breen GAM, Scheffler IE. Respiration-deficient Chinese hamster cell mutants: genetic characterization. Somat Cell Genet 5:225–240, 1979.
18. Guilbault GG, Kramer DN. Ultra sensitive, specific method for cyanide using p-nitrobenzaldehyde and o-dinitrobenzene. Anal Chem 28:834–836, 1966.
19. Scheffler I. Carbohydrate Metabolism in Cultured Cells. New York: Plenum Press, pp77–109, 1986.

20. Tickoo S, Russell S. *Drosophila melanogaster* as a model system for drug discovery and pathway screening. Curr Opin Pharmacol 2:555–560, 2002.
21. Demerec M. Biology of Drosophila. New York: John Wiley & Sons Inc, 1950.
22. Dow JT, Davies SA. Integrative physiology and functional genomics of epithelial function in a genetic model organism. Physiol Rev 83:687–729, 2003.
23. Agrawal N, Pallos J, Slepko N, Apostol BL, Bodai L, Chang LW, Chiang AS, Thompson LM, Marsh JL. Identification of combinatorial drug regimens for treatment of Huntington's disease using Drosophila. Proc Natl Acad Sci U S A 102:3777–3781, 2005.
24. Manev H, Dimitrijevic N. Drosophila model for in vivo pharmacological analgesia research. Eur J Pharmacol 491:207–208, 2004.
25. Mushett CW, Kelley KL, Boxer GE, Richards JC. Antidotal efficacy of vitamin B12a (hydroxo-cobalamin) in experimental cyanide poisoning. Proc Soc Exp Biol Med 81:234–237, 1952.
26. Posner MA, Tobey RE, McElroy H. Hydroxocobalamin therapy of cyanide intoxication in guinea pigs. Anesthesiology 44:157–160, 1976.
27. Hall AH, Rumack BH. Hydroxycobalamin/sodium thiosulfate as a cyanide antidote. J Emerg Med 5:115–121, 1987.
28. Brouard A, Blaisot B, Bismuth C. Hydroxocobalamine in cyanide poisoning. J Toxicol Clin Exp 7:155–168, 1987.
29. van Buuren KJ, Nicholis P, van Gelder BF. Biochemical and biophysical studies on cytochrome aa 3. VI. Reaction of cyanide with oxidized and reduced enzyme. Biochim Biophys Acta 256:258–276, 1972.
30. Panda M, Robinson NC. Kinetics and mechanism for the binding of HCN to cytochrome-c oxidase. Biochemistry 34:10009–10018, 1995.
31. Abou-Seif MA. Blood antioxidant status and urine sulfate and thiocyanate levels in smokers. J Biochem Toxicol 11:133–138, 1996.
32. Hasuike Y, Nakanishi T, Moriguchi R, Otaki Y, Nanami M, Hama Y, Naka M, Miyagawa K, Izumi M, Takamitsu Y. Accumulation of cyanide and thiocyanate in haemodialysis patients. Nephrol Dialysis Transplant 19:1474–1479, 2004.
33. Zhang R, Shen Z, Nauseef WM, Hazen SL. Defects in leukocyte-mediated initiation of lipid peroxidation in plasma as studied in myeloperoxidase-deficient subjects: systematic identification of multiple endogenous diffusible substrates for myeloperoxidase in plasma. Blood 99:1802–1810, 2002.
34. van Dalen CJ, Kettle AJ. Substrates and products of eosinophil peroxidase. Biochem J 358:233–239, 2001.
35. Scanlon CE, Berger B, Malcom G, Wissler RW. Evidence for more extensive deposits of epitopes of oxidized low density lipoprotein in aortas of young people with elevated serum thiocyanate levels. PDAY Research Group. Atherosclerosis 121:23–33, 1996.
36. Exner M, Hermann M, Hofbauer R, Hartmann B, Kapiotis S, Gmeiner B. Thiocyanate catalyzes myeloperoxidase-initiated lipid oxidation in LDL. Free Radic Biol Med 37:146–155, 2004.
37. Carterson AJ, Morici LA, Jackson DW, Frisk A, Lizewski SE, Jupiter R, Simpson K, Kunz DA, Davis SH, Schurr JR, Hassett DJ, Schurr MJ. The transcriptional regulator AlgR controls cyanide production in *Pseudomonas aeruginosa*. J Bacteriol 186:6837–6844, 2004.
38. Kondo H, Binder MJ, Kolhouse JF, Smythe WR, Podell ER, Allen RH. Presence and formation of cobalamin analogues in multivitamin-mineral pills. J Clin Invest 70:889–898, 1982.
39. Kanazawa S, Terada H, Iseki T, Iwasa S, Okuda K, Kondo H, Okuda K. Binding of cobalamin analogs to intrinsic factor-cobalamin receptor and its prevention by R binder. Proc Soc Exp Biol Med 183:333–338, 1986.
40. Fedosov SN, Berglund L, Fedosova NU, Nexo E, Petersen TE. Comparative analysis of cobalamin binding kinetics and ligand protection for intrinsic factor, transcobalamin, and haptocorrin. J Biol Chem 277:9989–9996, 2002.
41. Ermens AA, Vlasveld LT, Lindemans J. Significance of elevated cobalamin (vitamin B12) levels in blood. Clin Biochem 36:585–590, 2003.
42. Fedosov SN, Petersen TE, Nexo E. Binding of cobalamin and cobinamide to transcobalamin from bovine milk. Biochemistry 34: 16082–16087, 1995.
43. Kolhouse JF, Kondo H, Allen NC, Podell E, Allen RH. Cobalamin analogues are present in human plasma and can mask cobalamin deficiency because current radioisotope dilution assays are not specific for true cobalamin. N Engl J Med 299:785–792, 1978.
44. el Kholty S, Gueant JL, Bressler L, Djalali M, Boissel P, Gerard P, Nicolas JP. Portal and biliary phases of enterohepatic circulation of corrinoids in humans. Gastroenterology 101:1399–1408, 1991.
45. Kondo H, Kolhouse JF, Allen RH. Presence of cobalamin analogues in animal tissues. Proc Natl Acad Sci U S A 77:817–821, 1980.
46. Baker H, Frank O, Khalil F, DeAngelis B, Hutner SH. Determination of metabolically active B12 and inactive B12 analog titers in human blood using several microbial reagents and a radiodilution assay. J Am Coll Nutr 5:467–475, 1986.
47. Kolhouse JF, Allen RH. Absorption, plasma transport, and cellular retention of cobalamin analogues in the rabbit. Evidence for the existence of multiple mechanisms that prevent the absorption and tissue dissemination of naturally occurring cobalamin analogues. J Clin Invest 60:1381–1392, 1977.
48. Kondo H, Iseki T, Goto S, Ohto M, Okuda K. Effects of cobalamin, cobalamin analogues and cobalamin binding proteins on P388D1 mouse leukemic cells in culture. Int J Hematol 56:167–177, 1992.
49. Weinberg JB, Shugars DC, Sherman PA, Sauls DL, Fyfe JA. Cobalamin inhibition of HIV-1 integrase and integration of HIV-1 DNA into cellular DNA. Biochem Biophys Res Commun 246:393–397, 1998.
50. Coates ME, Davies MK, Dawson R, Harrison GF, Holdsworth ES, Kon SK, Porter JW. The activity for chicks of some vitamin B12-like compounds. Biochem J 64:682–686, 1956.
51. Stabler SP, Brass EP, Marcell PD, Allen RH. Inhibition of cobalamin-dependent enzymes by cobalamin analogues in rats. J Clin Invest 87: 1422–1430, 1991.