Toxicologic Studies on the Avicide 3-Chloro-p-toluidine

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Toxicologic Studies on the Avicide 3-Chloro-p-toluidine. FELSENSTEIN, W. C., SMITH, R. P. AND GOSSELIN, R. E. (1974). Toxicol. Appl. Pharmacol. 28, 110-125. 3-Chloro-p-toluidine (3-CPT) [also known as 3-chloro-4-methylaniline (3-CMA)] induces methemoglobinemia when given in lethal doses to mice and rats, but not when given to chickens. Death in laboratory rodents, however, appears to be unrelated to the methemoglobinemia. Peak concentrations of methemoglobin were lower than those generated by other compounds such as sodium nitrite or p-aminopropiophenone. Peak values were reached within the first hour but death occurred after several hours in rats, or several days in mice, when circulating methemoglobin concentrations declined toward normal. Methylene blue attenuated the methemoglobinemic response to 3-CPT, but increased the mortality. Hyperbaric oxygen increased methemoglobin concentrations after 3-CPT, but had no effect on mortality. Pretreatment with α-naphthylisothiocyanate (ANIT) decreased methemoglobin concentrations in mice after 3-CPT, but had no effect on mortality. Mortality in mice after 3-CPT was increased by cold stress or contemporaneous administration of Dial-Urethan. A profound and persistent hypothermia in mice and rats occurred after 3-CPT, and mice showed a dramatic but transient decrease in pulmonary ventilation. Elevated ambient temperatures partially blocked the hypothermic response, but, at least in mice, warming did not prevent death. Blood concentrations of 3-CPT in fatally poisoned mice appeared to decrease linearly with time. The half-life was estimated as 6.6 hr. Low concentrations of methemoglobin persisted for at least 24 hrs, after which time Heinz bodies were found in peripheral erythrocytes. Rats appeared to be more sensitive to 3-CPT than mice, and in contrast to the latter exhibited a significant hemoconcentration. In species resistant to 3-CPT-methemoglobinemia, death appears to be due to an uncharacterized blocking of energy metabolism.

An interesting example of a relatively new pesticide with novel target specificity is the avicide, 3-chloro-4-methylaniline (3-CMA). It has, however, been consistently referred to in the literature as 3-chloro-p-toluidine (3-CPT) and for that reason this more ambiguous name is retained here.

1 Supported by USPHS Grant HL 14127 from the National Heart and Lung Institute. A preliminary report of this work was presented at the meeting of the American Society for Pharmacology and Experimental Therapeutics, Burlington, Vermont, August, 1971: Abstract, Pharmacologist 13, 241 (1971).

2 Sponsored by Research Training Program, Perrine Primate Laboratory, Environmental Protection Agency.

3 Present address: Experimental Biology Laboratory Division, National Environmental Research Center, EPA, Research Triangle Park, North Carolina 27711.
When used either as the free base or as the hydrochloride salt, it is extremely toxic to certain species of birds. Its effectiveness against such feathered pests as starlings, crows and blackbirds was first demonstrated by workers at the Denver Wildlife Research Center, U. S. Department of the Interior (DeCino et al., 1966; Besser et al., 1967). They reported approximate acute oral LD50 values in mg/kg of 3.8 for starlings, 1.8–3.2 for red-winged blackbirds and 1.8 for crows. Much higher values were found for house sparrows (320–450) and Cooper's hawks (320–1000). The acute oral and intravenous LD50 values in starlings were virtually identical, indicating a very high degree of gastrointestinal absorption.

Toxic effects in birds and mammals have been described by Peoples (1965) and Apostolou and Peoples (1970, 1971), Mull et al. (1972), and Mull and Giri (1972). Based on findings of renal lesions and rapid rises in blood uric acid concentration, these investigators concluded that nephrotoxicity was the primary lethal effect in birds except when death occurred rapidly after large doses of 3-CPT. In a few cases species sensitivity showed a good correlation with renal N-deacetylase activity, suggesting that the nephrotoxicity was related to the presence of free 3-CPT. Methemoglobinemia was not observed in birds although it was inferred in several mammalian species. The oral toxicity in laboratory mammals approximates that of the less sensitive avian species above.

In mammals signs of central nervous system depression, muscular weakness and cyanosis occur at toxic doses. Tolerance was said to develop rapidly to repeated oral doses in rats and mice (Apostolou and Peoples, 1970). Methemoglobinemia was held accountable as the principal toxic effect of single doses in mammals. On parenteral administration to rats, however, the N-acetylated metabolite of 3-CPT had a toxicity equivalent to the parent compound even though it was inactive as a methemoglobin-former (Apostolou and Peoples, 1971). Moreover, a compound closely related to 3-CPT, p-aminotoluene, has only weak methemoglobin-generating activity in mice (Smith et al., 1967). This study was undertaken to assess the importance of the methemoglobinemia ascribed to 3-CPT as compared with other possible toxic effects.

**METHODS**

The hydrochloride salt of 3-CPT was obtained through the courtesy of the Denver Wildlife Research Center. It was used in all experiments without further purification because only single spots and peaks were observed on thin-layer and gas-liquid chromatography, respectively. When dissolved in distilled water for parenteral administration, the pH was 2.3. A solution of HCl adjusted to the same pH was sometimes used in control animals. Sometimes the latter produced mild, transient depression and tachypnea, but there were no permanent sequelae to as many as 5 daily injections in mice.

For *in vitro* experiments human blood samples drawn into K$_2$EDTA or heparinized blood samples from animals were used; red cells were washed with Krebs-Ringer-phosphate-glucose (KRPG) media (Smith, 1969) and resuspended to a final heme concentration of about 5.0 mM. In some experiments red cells were incubated for 1 hr at 37°C with 6.75 mM sodium nitrite to generate high concentrations of methemoglobin. The cells were then washed again and resuspended in KRPG.
Total hemoglobin was determined spectrophotometrically as cyanmethemoglobin, hematocrits were measured with a microcentrifuge and reader, methemoglobin was determined by a spectrophotometric technique (Smith, 1971), and Heinz bodies were visualized by a vital staining method (Beutler et al., 1955). Phenylhydrazine was used to generate Heinz bodies in vitro for comparative purposes. Blood oxygen capacity was determined manometrically (Smith, 1967). Colonic temperatures were taken with thermometer probes.

Tissues obtained from animal autopsies were fixed in formalin or Bouin's solution and stained with hematoxylin and eosin. A special in vivo method of fixation (Malm, 1962) was used to prepare the brain of 1 of the few mice that developed diffuse neurologic signs including marked intention tremors, but the histologic examination was negative.

Statistical comparisons were based on standard Student's t or Chi-square tests. The computer program of Spratt (1966) provided LD50 values. Groups of at least 10 mice were used at each dose level.

**Chickens.** Adult Rhode Island hens were obtained locally. Although some birds were found to have mycoplasma infections, it is unlikely that this affected our observations. Doses of 3-CPT in a volume of 1 ml/kg were given by the wing vein, and blood samples were taken by cardiac puncture.

**Mice.** Female Swiss Webster mice, 25–35 g, were used. All drugs and chemicals were given ip as aqueous solutions (10 ml/kg), except for α-naphthylisothiocyanate (ANIT) which was dissolved in corn oil and given by gavage, and the commercial parenterals, Dial-Urethan and pentobarbital (Nembutal), which were injected undiluted. Blood samples were taken from the tail or by decapitation. Pulmonary ventilation was studied in a small animal body plethysmograph designed by Fairchild (1972). Hexobarbital sleeping times were measured by the protocol of Axelrod et al. (1954). In acute hyperbaric exposures (4 ATA or 45 PSIG of 100% oxygen), a 2-liter metal chamber was used. In the 7-day exposures animals were maintained under 100% oxygen at ambient pressures or under 0.5 ATA of air in a vacuum chamber.

**Rats.** These were 250–300 g females of the Long-Evans strain. Intraperitoneal injection volumes were kept at 5 ml/kg. Blood samples were taken either from the portal vein after laparotomy under ether anesthesia or from a cannula placed in the carotid artery under pentobarbital anesthesia.

**Cats.** Adult animals were anesthetized with pentobarbital and 1 carotid artery was cannulated for blood pressure measurements and blood sampling. Injections were made via a cannula in the femoral vein. Neuromuscular junction effects were examined with a somatic nerve (peroneal) and tibialis anterior muscle preparation. Repetitive supramaximal stimuli were applied to the peripheral cut end of the nerve. Effects on ganglionic transmission were monitored contemporaneously through the activity of a nictitating membrane responding to periodic supramaximal stimulation of the distal cut end of the ipsilateral cervical sympathetic chain below the superior cervical ganglion. Respiration was monitored with a pneumatic cuff, and electrocardiograms (standard lead II) were obtained with subcutaneously implanted needle electrodes. Injections of 3-CPT were intermittent in a constant volume of 1 ml or by infusion at various rates and concentrations.

**Determination of 3-CPT.** An analytical method described by J. Henry (personal communication) was modified to permit the microdetermination of 3-CPT in blood.
The color reaction is based on diazotization with nitrite in protein-free filtrates followed by coupling with N-naphthylethylenediamine. The lower limit of sensitivity is about 5 µg/ml. Details of this method as well as applications of thin-layer and gas-liquid chromatographic techniques to blood and other body fluids are to be published elsewhere.

RESULTS

Chickens

3-CPT given iv to chickens in lethal doses (0.028–0.084 mmol/kg) produced transient signs of weakness, depression and a presumptive tachypnea with apparent recovery after several hours. Death occurred unexpectedly hours to days later, the interval to death varying inversely with the dose. Although not directly observed during life, post-mortem examination of the stomach of 3 birds indicated feeding until shortly before death. There were no consistent gross anatomic lesions, although the kidneys appeared congested or hemorrhagic in 2 instances. Histologically (postmortem autolytic changes limited satisfactory preparations to only 1 dosed bird) renal tubular degenerative changes were observed with distortion of architecture, decreased staining of nuclei, and mixed interstitial round cell infiltration. These changes did not appear severe enough to be compatible with a lethal nephrotoxic action.

Control values for methemoglobin in chicken blood averaged 7%. Despite this high normal concentration, as shown in Fig. 1, chicken red cells in KRPG are neither remarkably sensitive nor resistant to the methemoglobin-generating activity of sodium nitrite, when compared with feline, canine or human red cells tested under similar conditions (Stolk and Smith, 1966). The response of methemoglobinemic hen cells to 10⁻⁵ M methylene blue is also similar to that of mammalian red cells (Fig. 1).

When assayed in blood samples taken 40 min after the administration of lethal doses of 3-CPT, methemoglobin concentrations were not significantly different from those of
controls. Heinz bodies were not found. Indeed, incubation of the nucleated chicken erythrocytes with phenylhydrazine (Beutler, 1955) also failed to produce Heinz bodies.

**Mice**

The ip LD50 for 3-CPT (hydrochloride salt) was 1.9 mmol/kg with 95% confidence limits of 1.7 to 2.1. After single injections of toxic doses (1–2 mmol/kg) mice exhibited the following signs: prompt decline in pulmonary ventilation within 1 min, limb weakness which progressed to loss of the righting reflex with concomitant spinal hyperreflexia of the hind limbs. The corneal reflex remained intact. Altered respiration and loss of the righting reflex appeared prior to a moderate cyanosis and were transient, usually lasting less than 2 hr. Thereafter, the animals continued to show mild hind limb weakness, general depression (lethargy, anorexia), cyanosis, ruffled fur and occasionally tremors intensified by voluntary muscle activity.

Rarely, monoplegia of a hind limb occurred acutely and recovered slowly over 2–3 days. Reduced blood pressure was suggested by decreased force of bleeding. Urine production was apparent at 24 hr, and urine pH and content of protein, glucose, ketoncs, bilirubin and blood (Bili-Labstix) were all normal at that time. Administration of oxygen, even under hyperbaric pressure, did not diminish the cyanosis.

Death occurred quietly. Although the question could not be resolved definitely, the terminal event probably was cardiovascular collapse, rather than respiratory failure, i.e., cardiac activity was never detected after cessation of respiration. Convulsions did not occur. In surviving animals signs disappeared gradually over several days. Most deaths occurred between the second and fourth day although the time could be shortened by increasing the dose.

The acute effects of 3-CPT on pulmonary ventilation in mice are illustrated in Fig. 2. The fall in minute volume was prompt, severe and moderately prolonged. Statistically
significant effects are apparent at 30, 60 and 90 min. Isolated observations at later times suggest at least partial recovery in 3-CPT-dosed animals and a fatigue phenomenon in control animals probably resulting from the restraint imposed by the apparatus.

Gross and microscopic examination of the major organs revealed no consistent lesions. At times the kidneys exhibited degenerative changes, primarily of the convoluted tubules, especially after multiple sublethal doses. Interstitial hemorrhages and/or round cell infiltration were seen occasionally. Glomeruli were consistently spared. In general, the degree of pathologic change did not suggest a lethal nephrotoxic effect. Infrequently, the gross appearance of the lung was of congestion and even infarction, but it was normal in the majority of cases. Microscopically, the changes did not extend beyond variable degrees of interstitial cell infiltration with or without scattered focal hemorrhages. Again, the pathologic findings did not favor pulmonary failure as a cause of the cyanosis or death.

Initially, we believed the foamy vacuolization in the liver to be abnormal, especially since this finding was more prominent after repeated sublethal doses. Furthermore, there was apparent agreement with the appearance of reversible lesions described in the livers of 3-CPT-dosed rats which were thought to represent fat infiltration (Peoples, 1965). However, this histologic picture was also regularly encountered in the livers of normal controls of our strain of Swiss-Webster mice. This observation is not described in well known textbooks of veterinary histology or pathology (Cotchin and Roe, 1967; Smith and Jones, 1957). Pentobarbital sleeping times 24 hr after 1.9 mmol 3-CPT/kg were not significantly different from control. The same was true for blood glucose values after a 12-hr fast.

Although repeated injections regularly produced spleens that were quite dark in appearance, there was no clear cut correlation with the occasional microscopic finding of excessive pigment, presumably hemosiderin.

<table>
<thead>
<tr>
<th>TABLE 1</th>
</tr>
</thead>
<tbody>
<tr>
<td>METHEMOGLOBIN VALUES IN MICE AFTER THE INTRAPERITONEAL ADMINISTRATION OF 3-CPT</td>
</tr>
</tbody>
</table>

| Dose (mmol/kg) | Minutes after injection | Percent methemoglobin
<table>
<thead>
<tr>
<th></th>
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</thead>
<tbody>
<tr>
<td>1.0</td>
<td>10</td>
<td>27 ± 6</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>25 ± 5</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>19 ± 5</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>13 ± 5</td>
</tr>
<tr>
<td></td>
<td>120</td>
<td>13 ± 7</td>
</tr>
<tr>
<td></td>
<td>180</td>
<td>12 ± 4</td>
</tr>
<tr>
<td></td>
<td>24 hr</td>
<td>---</td>
</tr>
<tr>
<td>2.0</td>
<td>10</td>
<td>29 ± 7</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>36 ± 9</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>35 ± 7</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>28 ± 10</td>
</tr>
<tr>
<td></td>
<td>120</td>
<td>23 ± 9</td>
</tr>
<tr>
<td></td>
<td>180</td>
<td>24 ± 4</td>
</tr>
<tr>
<td></td>
<td>24 hr</td>
<td>9 ± 4</td>
</tr>
<tr>
<td>4.0</td>
<td>Methemoglobin values at death (6.7 ± 1 hr) were 9 ± 4%</td>
<td></td>
</tr>
</tbody>
</table>

* Values are means ± SD for 4–6 animals. Methemoglobin values in the absence of 3-CPT averaged 1.3 ± 0.6%.

The methemoglobinemic response of mice to 3-CPT is indicated by the data in Table 1. Unequivocal methemoglobin accumulation occurred at a dose of 1 mmol/kg. Increasing the dose to 2 mmol/kg led to an approximately proportional increase in methemoglobinemia. The time to peak methemoglobin concentrations was similar to that for
sodium nitrite (Smith and Layne, 1969), but low concentrations of methemoglobin were unusually persistent after 3-CPT. This persistence is also noted in the data of Smith et al. (1967) for other arylamino compounds, including the closely related \textit{p}-amino-toluene. Although a 2 mmol/kg dose is about the LD50 of 3-CPT, methemoglobin concentrations are well below those found to be lethal with other agents (Bodansky, 1951; Smith and Layne, 1969). Moreover, death did not usually occur for several days. A dose of 4 mmol/kg (Table 1) reduced the survival time to about 7 hr but methemoglobin values at that time were well below the peak concentrations generated by lower doses.

Persistence of a 3-CPT methemoglobinemia was even more apparent when sublethal doses (e.g., 1 mmol/kg) were given at 2–3-day intervals. With this regimen, there was no evidence of tolerance; instead, most animals succumbed after only a few doses whereas a single such dose produced only mild signs of intoxication.

As expected, 3-CPT did not generate methemoglobin when it was added to rabbit blood in vitro in concentrations of $1 \times 10^{-3}$ M. The persistence of the methemoglobinemia noted above suggested that 3-CPT or one of its metabolites may inhibit red cell methemoglobin reductase activity. Accordingly, 3-CPT in concentrations up to $1 \times 10^{-3}$ M was added to suspensions of rabbit erythrocytes made methemoglobinemic with sodium nitrite. Rates of methemoglobin reduction in such cases were not significantly different from control values.

Heinz bodies were detected in 3-CPT-poisoned mice, but they were not seen until after 30 hr. Consistently they appeared as 1–2 relatively large, round, deeply staining inclusions which differed in appearance from those produced in vitro in mouse cells with phenylhydrazine. There was no evidence for gross hemolysis: hematocrits remained stable (or at times increased) and plasma samples did not appear to contain hemoglobin. A subacute hemolytic process could not be ruled out because of the findings in spleens of mice that had survived for several days. Unidentified microscopic “debris” was seen in wet preparations of red cells. The oxygen transport capability of such blood, however, was not significantly impaired since the manometrically determined oxygen capacity agreed well with the spectrophotometrically determined total hemoglobin when corrected for the fraction present as methemoglobin.

As shown in Table 2, pretreatment of mice with methylene blue significantly attenuated the methemoglobinemic response to 3-CPT. In spite of this expected mitigating effect, the acute (3 hr) mortality due to 3-CPT was significantly increased by methylene blue. Among the methemoglobin-generating agents tested (Bodansky, 1951; Smith and Layne, 1969), unequivocal protection against death by methylene blue is the general rule. Admittedly, however, deaths were never seen within 3 hr after this combination of 3-CPT and methylene blue in unbled mice. The stress of bleeding may have shortened survival times here.

When mice were exposed to hyperbaric oxygen for either 40 or 180 min after the injection of 3-CPT, methemoglobin concentrations were significantly increased over those of animals kept in air at ambient pressure (Table 2). Similar results were obtained by Goldstein and Doull (1973) in rats with \textit{p}-aminopropiophenone. Despite the intensified methemoglobinemia, hyperbaric oxygen did not affect the acute mortality after 3-CPT. It appeared to shorten the acute depression phase of the intoxication, but other signs were not changed.

Since it was demonstrated to be inactive \textit{in vitro}, it is evident that a metabolite of
### TABLE 2

Effects of Various Pretreatments on Methemoglobinemia and Short-Term Survival of Mice after 3-CPT

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Percent methemoglobin</th>
<th>No. dead (3 hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Minutes after injection</td>
<td>10</td>
</tr>
<tr>
<td>Controls&quot;</td>
<td></td>
<td>29 ± 7</td>
</tr>
<tr>
<td>Methylene blue&quot;</td>
<td></td>
<td>14 ± 3</td>
</tr>
<tr>
<td>p value vs controls</td>
<td></td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Oxygen, 4 ATA&quot;</td>
<td></td>
<td>48 ± 12</td>
</tr>
<tr>
<td>p value vs controls</td>
<td></td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>ANIT&quot;</td>
<td></td>
<td>19 ± 7</td>
</tr>
<tr>
<td>p value vs controls</td>
<td></td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

" Injected with 2.0 mmol/kg 3-CPT only.

" Given ip 50 mg/kg 20 min before 3-CPT.

" Animals exposed for either 40 or 180 min after 3 CPT.

" Given by mouth in corn oil 80 mg/kg 48 hr before 3-CPT. A separate set of controls with corn oil was run for the ANIT experiment, but the results were not significantly different from those above.

3-CPT must be responsible for the methemoglobin formation. Attempts were made to modify the metabolism of 3-CPT by using a potent and long-lasting inhibitor of hepatic microsomal, drug-metabolizing enzymes, namely ANIT (Plaa et al., 1965). In our hands, the dose of ANIT given in Table 2 prolonged hexobarbital sleeping times in mice 7-fold over animals given corn oil alone. While the effect on 3-CPT methemoglobinemia was not nearly so dramatic (Table 2), definite attenuation of methemoglobinemia did result, although it was not statistically significant at all sampling times. ANIT had no effect on mortality after 3-CPT.

The effect of methylene blue on 3-CPT toxicity was explored in more detail; Fig. 3

![Fig. 3. Isobologram of 7-day LD50 values of methylene blue and 3-CPT alone and in combination with half the LD50 of the other in mice. The 95% confidence limits are bracketed. The dashed line is that for strict additivity of toxicity between the 2 chemicals.](image-url)
summarizes the results. The 7-day LD50 of intraperitoneally administered methylene blue was found to be 0.18 mmol/kg with 95% confidence limits of 0.14 to 0.20 compared to the previously cited value of 1.9 mmol/kg of 3-CPT. Most deaths after methylene blue also occurred between day 2 and 4. These data are plotted in Fig. 3 as an isobologram (Gaddum, 1959) where the dashed line indicates strict additivity of toxic effects. The LD50 for methylene blue was then determined when given with one-half the LD50 dose of 3-CPT and vice versa. These two points fall above the line of additivity and outside the triangle of potentiation of toxic effects. Although the combined toxicities of the 2 compounds appear less than additive, it is seen that the LD50 dose of 3-CPT was, in fact, significantly reduced (potency increased, \( p < 0.05 \)) in the presence of methylene blue. This was confirmed by probit analysis of potency ratios (Spratt, 1966).

### Table 3

<table>
<thead>
<tr>
<th>Oxygen tension</th>
<th>Air temperature casting</th>
<th>Sedation</th>
<th>No. dead</th>
<th>No. dosed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Air, 0.5 ATA</td>
<td>4</td>
<td>Dial</td>
<td>15/15</td>
<td>6/10</td>
</tr>
<tr>
<td>Air, 1.0 ATA</td>
<td>20</td>
<td>3-CPT</td>
<td>7/15</td>
<td>8/10</td>
</tr>
<tr>
<td>Oxygen, 1.0 ATA</td>
<td>32</td>
<td>Dial + 3-CPT</td>
<td>8/15</td>
<td>10/10</td>
</tr>
<tr>
<td>No significant differences</td>
<td>4 vs 20 ( p &lt; 0.01 )</td>
<td>3-CPT vs combination</td>
<td>0/6</td>
<td>6/6</td>
</tr>
</tbody>
</table>

* 3-CPT given ip 1.8 mmol/kg. All mortalities are for 7 days. Exposures to various oxygen tensions and ambient temperatures were continuous.

* Saline controls (not shown) carried under the same conditions. None of these died except for 2/10 mice exposed to oxygen at 1.0 ATA.

* Dial given ip 0.45 ml/kg 30 min before 3-CPT.

The influences of oxygen tension, ambient temperature and sedation on long-term (7 day) survival after 3-CPT are summarized in Table 3. Neither hypoxia nor hyperoxia as defined in Table 3 had a significant influence on mortality. Deaths among control mice after 5 days in 100% \( \text{O}_2 \) at ambient pressure were presumed to result from oxygen toxicity, which may also account for the somewhat higher mortality among 3-CPT animals under \( \text{O}_2 \). The toxicity of 3-CPT was strikingly potentiated by exposure to cold, as reflected both by a significantly increased mortality (Table 3) and a shorter time to death. Elevated ambient temperatures, however, did not protect against death. A sedative dose of Dial-Urethan increased mortality after 3-CPT. Animals given the combination never regained their righting reflex, and again the time to death was shorter than with 3-CPT alone. Even here it was not clear that respiratory arrest was the terminal event.

A dramatic and long-lasting hypothermic effect of 3-CPT is shown by the data in Table 4. Colonic temperatures fell by as much as 11°C, and were significantly depressed as long as 48 hr after administration. An appropriate dose of Dial-Urethan can produce a similar early depression of body temperature, but the effect did not last beyond 2 hr.
<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose $^a$</th>
<th>Temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10</td>
<td>20</td>
</tr>
<tr>
<td>Controls</td>
<td>39 ± 0.3</td>
<td>40 ± 0.0</td>
</tr>
<tr>
<td>3-CPT</td>
<td>2.0</td>
<td>36 ± 0.6 $^b$</td>
</tr>
<tr>
<td>Dial-Urethan</td>
<td>0.6</td>
<td>34 ± 0.4 $^*$</td>
</tr>
<tr>
<td>Methylene blue</td>
<td>0.18</td>
<td>33 ± 1.0 $^b$</td>
</tr>
<tr>
<td>Aniline</td>
<td>2.0</td>
<td>35 ± 0.6 $^b$</td>
</tr>
</tbody>
</table>

$^a$ Given ip. Dose as mmol/kg except in the case of Dial-Urethan, where it is ml/kg.

$^b$ Significantly different from controls ($p < 0.01$).

$^c$ Mean for 2 surviving animals. All other results are means ± SD for 3 or 4 animals.
Methylene blue also produced hypothermia, but in animals that survived, body temperature returned to normal in 24 hr. Aniline, at a dose equimolar to that of 3-CPT, produced a transient hypothermia of smaller magnitude. Instead of depression, aniline produced tremors and other signs of neuromuscular hyperactivity.

During severe 3-CPT hypothermia, warming the ambient environmental temperature to 32°C raised colonic temperatures by increments as large as 7°C within 30 min without evidence of thermal overshoot. In such cases the mice became much more active and ruffling of fur (piloerector activity) disappeared although cyanosis persisted. When returned to room temperature, the colonic temperatures fell to previous hypothermic levels, but they appeared to do so much more slowly. Similarly, when mice were placed in a warm environment prior to dosing with 3-CPT, the severity and rate of fall in body temperature were greatly diminished. The acute signs were also markedly altered with attenuation and shortening of the depressive phase, which was now replaced by hyper-irritability and hyperreactivity with a suggestion of impending exhaustion.

Data on blood concentrations of 3-CPT after ip administration to mice of 2.0 mmol/kg are summarized in Fig. 4. The dashed line indicates the least squares fit of all the data except that at 24 hr. The data fit this linear plot much better than the more conventional logarithmic one.

Rats

A series of rats were injected with 3-CPT to compare their responses with those of other species. Rats were more sensitive than mice to the 2.0 mmol/kg dose. There was a rapid onset of apparent tachypnea with gasping, marked bilateral hind limb weakness, a deeper state of coma than exhibited by mice and loss of pain reflexes after a brief period.
of hyperreflexia. Pallor was the rule rather than cyanosis. Rats regained spontaneous activity after 1–2 hr, but hind limb paresis persisted. After another hour, righting reflexes were lost again and the animals seemed to pass into a state of collapse with shallow stertorous respirations, which ceased prior to cessation of heart action in a few cases.

Data on methemoglobin concentrations, hematocrit and colonic temperatures in rats are summarized in Fig. 5. Methemoglobin values were not so high as in mice, which may be the reason for the lack of cyanosis. An effect not observed in mice was a consistent and significant hemoconcentration, as indicated by the steadily rising hematocrit.

![Graphs showing methemoglobin, hematocrit, and colonic temperature changes over time.](image)

**Fig. 5.** Methemoglobin concentration, hematocrit and colonic temperature in rats given 2.0 mmol/kg or 0.6 ml/kg of Dial-Urethan ip. Statistical comparisons were based on at least 6 animals, and asterisks indicate mean values significantly different from preinjection controls ($p < 0.05$).

Hypothermia was also a feature of 3-CPT intoxication in rats although colonic temperatures did not fall as far as in mice. In rats the hypothermic response closely paralleled that produced by a near-lethal dose of Dial-Urethan, but in contrast to mice, no rat survived this dose of 3-CPT for longer than 6 hr.

**Cats**

In anesthetized cats given successive iv doses of 3-CPT, unequivocal circulatory collapse occurred after a total of 36 mg/kg. On the other hand, during continuous iv infusion, with a dose rate increasing from 26 to 127 mg/kg/hr, respiratory depression occurred before significant falls in the blood pressure or persisting electrocardiographic
abnormalities. The latter were manifest by progressive changes consisting of diminishing R wave, elevation of the S-T segment, appearance of a Q-S wave, followed by reappearance of an abnormal R wave with extreme elevation of the S-T segment. Terminally, there was inversion of the T-wave, ventricular tachycardia, terminating in ventricular fibrillation. The earlier abnormalities were reversible on at least one occasion by administration of oxygen, but the concentration of methemoglobin at this time was 55%. The cumulative acute lethal dose after institution of respiratory assistance was 162 mg/kg.

No evidence was obtained for effects on ganglionic transmission, and although a slight neuromuscular blocking activity was seen, it certainly could not account for death. In cats, as well as mice, 3-CPT in blood was detectable only in the plasma phase.

**DISCUSSION**

Experimental results presented here show clearly that methemoglobinemia after lethal doses of 3-CPT in mice and rats is not the cause of death. Indeed, methemoglobinemia appears to play only a minor role in the signs of 3-CPT intoxication in these species. Not only were the methemoglobin values generated by lethal doses of 3-CPT (Table 1, Fig. 5) well below levels considered fatal when generated by other agents (Bodansky, 1951; Smith and Layne, 1969), but attenuation of the methemoglobinemia by methylene blue was accompanied by an increase in mortality (Table 2). Conversely, the phase of acute central nervous system depression appeared to be attenuated with hyperbaric oxygen despite the increase in methemoglobinemia.

This conclusion is consistent with observations made on rats by Lester et al. (1944) and those made on mice by Smith et al. (1967). Both found that methemoglobin concentrations in excess of 50% as generated by other arylamino compounds resulted in few or transitory symptoms. Lester et al. (1944) pointed out the lack of correlation between lethal doses of several arylamino compounds including aniline and the methemoglobin values at the time of death.

The above conclusion, however, cannot be drawn for cats, which are especially susceptible to methemoglobin formation by aniline and its congeners (Lester, 1944; Reiter, 1948). Perhaps this species difference is related to differences in the rates of conjugation of the free amine group. Humans conjugate arylamines with much greater facility than do cats (Welch et al., 1966). Methemoglobin values of at least 55% were reached during slow iv infusion of 3-CPT into cats. The contemporaneous respiratory depression and electrocardiographic abnormalities were both reversed by oxygen administration, suggesting that they were of anoxic origin secondary to the methemoglobinemia. This observation is in accord with that of Clark et al. (1943) after extensive studies of aniline toxicity in dogs. Dogs do not form methemoglobin after aniline as readily as cats, but they are much more sensitive than rodents (Spicer, 1950; Lester, 1944).

It is likely that the prolonged methemoglobinemia seen in mice after 3-CPT (Table 1) is a reflection of the slow decline in blood concentrations of the compound or a diazotizable metabolite (Fig. 4). Similarly, the increased methemoglobin formation after hyperbaric oxygen (Table 2) probably results from a prolonged or increased blood concentration of the amine form of 3-CPT as was found for p-aminopropiophenone by Goldstein and Doull (1973).

Obviously, methemoglobinemia plays no role in the signs seen in chickens poisoned
with 3-CPT since methemoglobin values were not different from those of controls. Chicken red cells were not unusually sensitive or resistant to nitrite (Fig. 1) despite normal methemoglobin concentrations of 7%, a figure higher than that for man and laboratory mammals (Bodansky, 1951). They were, however, resistant to the formation of Heinz bodies by phenylhydrazine, and chicken red cells may represent a useful system to study the postulated relationship between methemoglobin and Heinz body formation (Jandl et al., 1960).

The lethal mechanism of 3-CPT in birds and mammals has yet to be precisely defined. In the former our observations were limited. The early transient signs of apparent tachypnea, depression and muscle weakness followed by a period of apparent recovery prior to death corresponded closely to the description of Mull et al. (1972). However, the lack of significant renal microscopic lesions and the apparent well-being of the birds until their sudden death make it difficult to accept renal failure as the exclusive lethal event. Nor did renal failure appear to play a role in poisoned mice. Our observations suggest that 3-CPT has complex, but different, toxic effects in mice, rats and cats. It appears to resemble aniline, which has been extensively studied for more than a century without a clear elucidation of its toxic mechanism in species resistant to its methemoglobin-generating activity. Gross and histologic examination of the major thoracic and abdominal viscera of mice dying at various times after single or repeated doses of 3-CPT failed to reveal consistent or significant abnormalities. We believe that the hepatic cell "vacuolization" suggestive of fatty infiltration is a frequent, if not constant, finding in normal animals.

The central and peripheral nervous system effects observed in mice were all transient. The occasional hindlimb monoplegias observed after intraperitoneal 3-CPT were presumably due to a local neurotoxic effect on the lumbrosacral plexus. A local irritant effect of the injected solution was not responsible since monoplegia was not produced by hydrochloric acid solutions of the same pH. The general muscle weakness seen in mice, but which was most prominent in rats, is not readily explained. It persisted well beyond the period of central nervous system depression and hypoxia ascribable to the methemoglobinemia. Muscle weakness was noted by Young et al. (1926) and Clark et al. (1943) in their studies on aniline toxicity. We found no significant neuromuscular blocking activity of 3-CPT in our studies with cats.

No single abnormal blood pigment accounts satisfactorily for the striking cyanosis. Cyanosis persisted long after peak methemoglobin values. Inadequate oxygenation of hemoglobin is unlikely since neither hypoxia nor hyperoxia had an effect on mortality (Table 3), and even hyperbaric oxygen failed to relieve the cyanosis. Despite the marked decline in pulmonary ventilation (Fig. 2), even at the lowest volume recorded (7 ml/min), the oxygen supplied is well in excess of the basal requirement of 1.5 ml/g per hr (Morrison, 1948). Significant amounts of other inert pigments, such as sulfhemoglobin, could not be demonstrated.

Vascular collapse could account for cyanosis, the absence of significant effects of oxygen (above) and the slow decline in 3-CPT blood concentrations. Direct measurements of the cardiovascular effects of 3-CPT in rodents to parallel those made in cats would be desirable. As with the case with Young et al. (1926) in observations on aniline-poisoned rabbits, we could not determine whether mice died in cardiovascular or respiratory failure.
Some changes reported by Mull et al. (1972) in chickens and some observed by us in rodents are compatible with the syndrome of acute adrenal failure: cyanosis due to vascular collapse, lethargy and muscle weakness, hypovolemia with rising hematocrit and hypothermia. The absence of a rise in eosinophil count and no change in serum electrolytes in Mull’s report and normal fasting blood glucose values as reported here, however, are not consistent with that hypothesis.

Another parallelism with the observations of Young et al. (1926) is the profound hypothermia induced in mice (Table 4) and rats (Fig. 5) by high doses of 3-CPT. The degree of hypothermia found is not lethal per se to either mice or rats since both are able to tolerate lower core temperatures (Herrington, 1940). Because these species depend greatly on metabolic heat production for the maintenance of body temperature, the hypothermia after 3-CPT is most likely due to an interference with metabolism. Impaired energy metabolism might explain why the lethargy and muscle weakness in poisoned animals are reversed if the ambient temperature is raised although the ultimate mortality is not changed. This hypothesis is consistent with increased mortality in a cold environment (Table 3), increased mortality in combination with methylene blue and stress (Table 2), and increased mortality in combination with Dial-Urethan (Table 3). The latter pretreatments alone also cause body temperatures to decline, presumably by different mechanisms. Hypothermia in mice after 3-CPT, unlike deep sedation, is not due to an impairment of the central thermoregulatory center. Piloerection does not occur during hypothermia from deep sedation, but it was present in 3-CPT-poisoned animals and disappeared on external warming. Certainly, an uncoupling of oxidative phosphorylation is not consistent with these observations.

If this presumed metabolic blockage is produced in varying degrees by aniline and its congeners, its contribution to the total toxicity of the compound might relate inversely to the methemoglobin-generating activity in a given species.

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