The Effect of Methylxanthines on the Hypocoagulability Induced by Chloroform in the Dog

By John B. Field, Ph.D., M.D., L. Graf, Ph.D. and Karl Paul Link, Ph.D.

Several attempts have been made to prevent or ameliorate the toxic effects and pathologic changes resulting from poisoning with chloroform or carbon tetrachloride. An account of these studies is reviewed by Neale and Winter. It has been shown that the degenerative changes in the liver of dogs and rats, resulting from prolonged exposure to chloroform or carbon tetrachloride can be prevented by feeding liver concentrates and fractions thereof. Forbes and associates, in a series of reports have demonstrated that one of the active principles present in a protective liver fraction was sodium xanthine. They also showed that the subcutaneous administration of sodium xanthine and guanine would protect against the hepatic damage. Neale and Winter reported that other purine substances as nucleic acid, guanosine and hypoxanthine would also prevent the pathologic degeneration and they suggested that the pyrimidine portion of the xanthine molecule as represented by uracil, was the active moiety.

We have previously reported that the methylxanthines, caffeine, (1,3,7-trimethylxanthine), theobromine, (3,7-dimethylxanthine) and theophylline (1,3-dimethylxanthine) when given orally, induce a state of hyperprothrombinemia and reduce the extent of the hypoprothrombinemia induced in the dog and rat by Dicumarol. A lesser protection

* However, Ravidin et al. and Calder have presented convincing evidence that the protective action observed following the subcutaneous administration of xanthine and its derivatives, was intimately related to its insolubility and the ensuing foreign body inflammatory reaction. Thus, even suspensions of carbon and red blood cells afforded a strong effect when introduced subcutaneously.

† Dicumarol is the trademark of the anticoagulant.

‡ A prolonged polemic was set off when a report by Quick denied the validity of the findings. Until the present only one other group has undertaken any substantial animal investigation. McCormick and Young not only demonstrated a reproducible hyperprothrombinemia induced by methylxanthines but also a substantial increase in A" globulin. Results in humans have been mixed.
was obtained with other purine compounds, xanthine (2,6-dioxypurine) and its
derivatives. The present report further indicates that the oral administration
of the methylxanthines (caffeine, theobromine, theophylline), adenine (6-amino-
) and several related compounds reduce the extent of the prothrombin and fibrinogen deficiency which follow a chloroform intoxication in dogs. A preliminary summary appeared in 1945.

METHODS

These studies were performed on normal mature dogs maintained on a regular stock ration of skim milk powder 40, yellow corn 15, meat scraps 15, wheat bran 10, wheat middlings 10, alfalfa meal 7, bone meal 2, and salt 1, or the commercial preparation known as Friskies.* In general, the technics were the same as those described in previous publications.†‡

Variation in absorption of the anesthetic, which is usually administered by inhalation, was overcome by giving it by stomach tube. Food was withheld from the dogs for 12 hours and then the desired quantity of chloroform in oil (4 ml. chloroform in 10 ml. solution with cotton seed oil) was given. Blood samples were withdrawn previous to the administration of chloroform and daily thereafter until the normal pre-test prothrombin and fibrinogen levels were restored. Each animal was rested for at least 2 to 3 weeks before a new experiment was begun.

The supplements were fed in gelatin capsules, in consecutive daily doses, beginning two days prior to administration of the chloroform, and were continued until a total of 5 doses were given.

EXPERIMENTAL

Prothrombin Time of Dogs Given Chloroform

The data are given in terms of 12.5 per cent plasma (1 part plasma, 7 parts saline solution) for reasons previously recorded, although whole plasma (100 per cent), and the 25 per cent plasma concentrations were routinely explored for prothrombin level (or activity). By feeding either a single dose of 4, 6, or 10 ml. or 2 consecutive daily doses of 2 ml. of chloroform, the prothrombin time of dog plasma (all dilutions) was significantly prolonged. Usually the hypoprothrombinemia persisted for 3 to 5 days (table 1). Individual animals differed widely in their response to identical treatment, but the hypoprothrombinemia (extent and duration) obtained with equal doses of chloroform on successive trials in the same dog was reproducible. By resting the animal at least two weeks between trials, the tendency towards an increased susceptibility (expressed by a more acute prothrombin deficiency) was reduced.

Effect of Methylxanthines on the Prothrombin Time of Dogs Given Chloroform

Representative results realized with individual dogs are given in table 1. When dogs standardized to a fixed dose of chloroform were fed daily doses of 100 mg. per kilo of caffeine, theobromine and theophylline† significant prolongations of the prothrombin time (all dilutions) were not observed (table 1). Most dogs exhibited only a transitory hypoprothrombinemia; some were almost completely

* Made by the Carnation Milk Company.
† The protective capacity of theophylline is less than that of caffeine and theobromine. Theophylline likewise has less protective action than caffeine or theobromine against the hypoprothrombinemia induced by 3,3'-methyleneph (4-hydroxycoumarin).‡
protected (table 1). These findings were reproduced in some 30 individual tests performed on 12 different dogs.

**Table 1.—Representative Effect of Various Xanthines on the Hypoprothrombinemia Induced by Giving Dogs Chloroform Orally**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Prothrombin time in seconds of 12.5 per cent plasma</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Normal 1 day after chloroform 2 days after chloroform 3 days after chloroform 4 days after chloroform 5 days after chloroform</td>
</tr>
<tr>
<td>2 ml. chloroform for 2 days (control)</td>
<td>21 56 92 84 46 32</td>
</tr>
<tr>
<td>2 ml. chloroform for 2 days + theobromine†</td>
<td>22 33 32 23</td>
</tr>
<tr>
<td>2 ml. chloroform for 2 days (control)‡</td>
<td>28 45 102 74 39 29</td>
</tr>
<tr>
<td>4 ml. chloroform, single dose (control)</td>
<td>27 70 66 34</td>
</tr>
<tr>
<td>4 ml. chloroform, single dose + caffeine†</td>
<td>24 21 27 32 23</td>
</tr>
<tr>
<td>6 ml. chloroform, single dose (control)</td>
<td>31 46 100 82 52 47</td>
</tr>
<tr>
<td>6 ml. chloroform, single dose + theophylline†</td>
<td>27 24 25 35 35 26</td>
</tr>
<tr>
<td>2 ml. chloroform for 2 days (control)</td>
<td>21 56 61 61 47 32</td>
</tr>
<tr>
<td>2 ml. chloroform for 2 days + xanthine†</td>
<td>23 20 31 76 51 31</td>
</tr>
<tr>
<td>4 ml. chloroform, single dose (control)</td>
<td>26 28 34 40 34 30</td>
</tr>
<tr>
<td>4 ml. chloroform, single dose + guanine†</td>
<td>21 25 36 41 42 31</td>
</tr>
</tbody>
</table>

* Each line of figures is a series of values obtained with a single animal, the test being performed on the same standardized animal.

† 100 mg. per Kg. daily dose given for 5 days beginning 2 days prior to chloroform administration.

‡ This control test was made 2 weeks after the normal prothrombin time was reached in the previous trial.

**Effect of Other Purines and Related Substances on the Prothrombin Time of Dogs Given Chloroform**

A wide variety of compounds related to the methylxanthines was also tested. Adenine afforded complete protection against the hypoprothrombinemia induced by chloroform. Creatine, creatinine, guanidine and uracil also gave some protection, but it was neither as consistent nor as complete as that afforded by the methylxanthines or adenine. On the other hand, little or no demonstrable protective action was obtained by feeding xanthine, guanine (see table 1), arginine, allantoin, uric acid or urea.
EFFECT OF METHYLXANTHINES ON HYPOCOAGULABILITY

Effect of Methylxanthines, Other Purines and Related Substances on the Fibrinogen Level of Dogs Given Chloroform*

Fibrinogen was determined by a standard colorimetric procedure21 on an aliquot of the plasma used for the prothrombin estimations. The quantity of chloroform that induces a detectable hypoprothrombinemia, simultaneously causes a moderate to drastic reduction in the plasma fibrinogen.16, 17 Although the reduction of fibrinogen in individual dogs differed, the qualitative response on successive trials in the same dog was very similar. Caffeine, theobromine, theophylline and adenine were effective in preventing a reduction in the fibrinogen

Table 2.—Representative Effect of Various Xanthines on the Fibrinogen Levels in Dogs Given Chloroform Orally*

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Plasma fibrinogen levels (mg. per cent)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Normal + S.D.</td>
</tr>
<tr>
<td>2 ml. chloroform for 2 days (control)</td>
<td>232 ± 6.7</td>
</tr>
<tr>
<td>2 ml. chloroform for 2 days + theobromine¶</td>
<td>173 ± 17.5</td>
</tr>
<tr>
<td>4 ml. chloroform, single dose (control)</td>
<td>256 ± 18.6</td>
</tr>
<tr>
<td>4 ml. chloroform, single dose + caffeine¶</td>
<td>178 ± 7.3</td>
</tr>
<tr>
<td>6 ml. chloroform, single dose (control)</td>
<td>148 ± 2.5</td>
</tr>
<tr>
<td>6 ml. chloroform, single dose + theophylline¶</td>
<td>156 ± 6.2</td>
</tr>
<tr>
<td>2 ml. chloroform for 2 days (control)</td>
<td>232 ± 6.7</td>
</tr>
<tr>
<td>2 ml. chloroform for 2 days + xanthine¶</td>
<td>249 ± 7.1</td>
</tr>
<tr>
<td>4 ml. chloroform, single dose (control)</td>
<td>253 ± 21.7</td>
</tr>
<tr>
<td>4 ml. chloroform, single dose + guanine¶</td>
<td>275 ± 15.7</td>
</tr>
</tbody>
</table>

* Each line of figures is a series of values obtained with a single animal, the test being performed on the same standardized animal.
† S.D., Standard Deviation.
¶ 100 mg. per kilo daily dose given for 5 days beginning 2 days prior to chloroform administration.

* The total plasma protein of dogs receiving chloroform was not materially altered under the conditions of the trials.
levels (table 2). Uracil showed a lesser protective action. However, creatine and creatinine did not prevent a reduction in plasma fibrinogen although they prevented the hypoprothrombinemia. Guanidine had a slight protective action, while guanine, xanthine, uric acid, allantoin, urea or arginine had no detectable action.

**Bromsulfalein Test in Dogs Given Chloroform**

The bromsulfalein retention test\(^2\) is based on the capacity of the normal liver to remove the dye, phenol tetrabromphthalein-disodiumsulfonate (Bromsulfalein, Hynson, Westcott and Dunning) from the blood stream within a limited period of time. In most forms of hepatic degeneration or intoxication, a reduced capacity to remove the dye from the blood stream exists. The dye in the plasma is measured colorimetrically by a standard procedure\(^2\). In the present experiments the degree of hepatic function by this method was routinely determined 2 days after administration of the chloroform.

When the dogs were given chloroform and one of the methylxanthines (caffeine, theobromine, theophylline) the capacity of the liver to remove the test dye from the circulation remained impaired, even though there was no detect-

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*We have found that the methylxanthines actually produce an increase in the plasma fibrinogen of dogs (see table 2). Therefore, the protective effect may only be a reflection of the elevated fibrinogen levels. (Increased Plasma Fibrinogen Induced by Methylxanthines, Field, J. B., Sveinbjornsson, A., and Link, K. P., J. Biol. Chem. 159: 525, 1945.) While the methylxanthines elevate plasma prothrombin and fibrinogen levels in the dog, rabbit and rat, adenine is one of several related compounds which possess only a borderline capacity to elevate the plasma fibrinogen in rabbits. This effect which is otherwise detectable with some difficulty, becomes more pronounced when the normal levels of prothrombin and fibrinogen are artificially reduced as reported herein.

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**Table 3—Representative Effects of Methylxanthines on the Removal of Bromsulfalein Dye from the Plasma of Dogs following Chloroform Liver Intoxication**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Per cent dye in plasma</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal dogs, (control)</td>
<td>10</td>
</tr>
<tr>
<td>6 ml. chloroform, single dose</td>
<td>85</td>
</tr>
<tr>
<td>6 ml. chloroform, single dose plus theobromine†</td>
<td>100</td>
</tr>
<tr>
<td>4 ml. chloroform, single dose</td>
<td>55</td>
</tr>
<tr>
<td>4 ml. chloroform, single dose plus caffeine†</td>
<td>50</td>
</tr>
<tr>
<td>10 ml. chloroform, single dose</td>
<td>85</td>
</tr>
<tr>
<td>10 ml. chloroform, single dose plus theophylline†</td>
<td>55</td>
</tr>
</tbody>
</table>

* The bromsulfalein dye (2 mg. per Kg.) was injected intravenously 2 days after feeding the chloroform.
† 100 mg. per Kg. daily dose given for 5 days beginning 2 days prior to chloroform administration.
able reduction in the prothrombin or fibrinogen levels. The results from many trials will be reported in a highly condensed form. The control experiments with normal dogs indicated that when a fixed quantity of dye (2 mg. per Kg.) was injected intravenously, only 5 to 20 per cent remained in the plasma over 5 minutes, and after 30 minutes none could be detected (table 3). When chloroform was given alone, and hepatic degeneration was apparent by an icteric plasma, from 50 to 100 per cent of the dye was determinable after 5 minutes, and 5 to 55 per cent after 30 minutes. In one dog following the supplemental feeding of 100 mg. per Kg. of caffeine daily, the administration of chloroform had no effect on the pre-test levels of plasma prothrombin (table 1). However, the plasma was icteric and 55 per cent of the test dye was detectable 5 minutes after injection and after 30 minutes. When 100 mg. per Kg. of theobromine was given in daily doses so that the plasma prothrombin and fibrinogen were unchanged after chloroform intoxication (the plasma was icteric), 60 per cent of the dye was present in the plasma after 5 minutes and 20 per cent could be detected after 30 minutes (table 3). Creatine also protected against chloroform hypoprothrombinemia. However, 30 per cent of the bromsulfalein dye was determinable in the plasma 5 minutes after injection and none after 30 minutes.

DISCUSSION

Our previous studies have indicated that caffeine, theobromine and theophylline given orally induce hyperprothrombinemia and elevate the plasma fibrinogen levels in various species. It was suggested that these effects probably indicated a functional stimulation of hepatic tissue. It appeared that the active purine bases more specifically affected the synthesis of prothrombin and fibrinogen than the other diversified functions of the liver. Previously this was supported by evidence that methylxanthines diminished the hypoprothrombinemia induced by the anticoagulant, dicumarol, and now this rationalization is strengthened by the demonstration that caffeine, theobromine, theophylline and adenine prevent the hypocoagulable manifestations produced by chloroform intoxication. This appears to occur even when liver destruction as revealed by icterus and the bromsulfalein function test was marked. Thus, highly icteric plasma and an impaired capacity of the liver to remove the dye from the circulation during chloroform intoxication occurred in the presence or absence of the purine bases. Caffeine, theobromine, theophylline and adenine did not appear to be particularly effective in reducing the noxious influence of chloroform on other normal functions of the liver. This observation is not without parallel since other workers have indicated that in the face of the multifarious character of hepatic activity, one or more normal functions may be inhibited or accelerated without necessarily interfering with the others.

The protective capacity of the other compounds merits comment. Creatine and creatinine protected standardized dogs against the experimental reduction in prothrombin but did not prevent the fibrinogen reduction. However, xanthine, arginine, allantoin, uric acid or urea were either markedly less effective or totally ineffective. These observations are not entirely in accord with those of Forbes and associates who found that after subcutaneous injection, xanthine and its analogues was effective in preventing the appearance of liver necrosis in rats.
given chloroform or carbon tetrachloride. However, by the subcutaneous route caffeine, theobromine, theophylline and adenine were so toxic that “it was difficult to test the relative protective activity.”5 P. 14

By giving the methylxanthines orally toxic symptoms were not encountered in this study and the nonspecific effects induced by subcutaneous inflammation were not observed.10

The work reported here and in other publications11 indicates that a definite relationship exists between the purine bases and at least those specific functions of the liver dealing with the elaboration of certain of the factors involved in the coagulation process.

Sykes et al.25 and Seegers26 have reported that chloroform liver damage in dogs associated with hypoprothrombinemia is accompanied by a pronounced decrease in A-globulin. With the demonstration that aminophyllin produces increased plasma A-globulin as well as hyperprothrombinemia in dogs14 support is given to the findings recorded here. It may be cogent to remind investigators who are lost in the intricacies of the delicate manoeuvre of considering the phenomenon of a state of hyperprothrombinemia25, 27 in both animals and humans, and who may or may not exercise their prerogative of utilizing the technic of diluted plasma, that more ready confirmation will probably result from tests of any agent under conditions of “prothrombin stress” and induced hypoprothrombinemia. Thus, clinicians debating the activity of the methylxanthines may benefit from the observation that Dr. John Olwin of Chicago26, p. 8 “commonly found it to be the case that far more Dicumarol is required when aminophyllin is also given.”

SUMMARY

1. The oral administration of a single or two consecutive daily doses of chloroform to dogs, induced hypoprothrombinemia and lowered the fibrinogen level. Both changes from the normal were most marked 48 hours after chloroform administration. By resting the dogs about 2 weeks between trials comparable reductions in the plasma prothrombin and fibrinogen levels were realized.

2. When caffeine, theobromine, theophylline and adenine were given orally to dogs prior to the oral administration of chloroform and continuing for five consecutive days, the hypoprothrombinemia was either markedly reduced or completely prevented. Creatine, creatinine, guanidine or uracil also afforded some protection. Guanine, xanthine, arginine, allantoine, uric acid or urea gave practically no protection.

3. Caffeine, theobromine, theophylline and adenine also prevented a reduction in the plasma fibrinogen level during chloroform intoxication. Uracil and guanidine showed some protective action, while creatine, creatinine, guanine, xanthine, uric acid, allantoine, urea and arginine gave no detectable protection.

4. Liver injury from the chloroform, as reflected by the bromsulfalein test and icteric plasma, was readily detectable when the methylxanthines or adenine were given with the chloroform, even though no change in prothrombin or fibrinogen level was apparent.

5. It appears that a definite relationship exists between the purine bases (caffeine, theobromine, theophylline and adenine) and the specific function of the
liver to elaborate plasma prothrombins and fibrinogen. The capacity of the liver
to synthesize prothrombin apparently can be affected independently of other
normal functions.

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