PROTHROMBIN DEFICIENCY AND THE BLEEDING TENDENCY IN LIVER INJURY (CHLOROFORM INTOXICATION)*

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Chloroform has been used extensively in experimental work for the production of liver injury. In pronounced intoxication there is extensive necrosis of the liver, and the animal shows marked disturbance of blood coagulation. Clots which form are flabby and are ineffectual in controlling hemorrhage from minor incisions. The work of Doyon (1) suggested that there is a decrease in the plasma fibrinogen. Whipple and Hurwitz (2) and Foster and Whipple (3) in careful quantitative studies confirmed this. Recently we have developed a method for the quantitative determination of prothrombin (4). With large chloroform doses we found not only a decrease in fibrinogen, but also a very marked fall in plasma prothrombin. The bleeding tendency in this condition is clearly due to a deficiency in both of these factors.

In the present paper we shall demonstrate that the prothrombin is more labile than the fibrinogen. When the chloroform dosage is small the prothrombin falls much more markedly than the fibrinogen. It is even possible by giving small repeated doses of chloroform to completely dissociate these two clotting factors. In such experiments, the fibrinogen level remains unaffected, but the prothrombin falls markedly, often with the development of a hemorrhagic tendency.

Methods

The technic of Jones and Smith (5) was used for the determination of plasma fibrinogen.

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Our prothrombin titration method (4) has been modified in certain particulars. The reagents now in use are:

1. Saline (0.9 per cent sodium chloride).

2. Oxalated Saline.—Dissolve 0.092 gm. $K_2C_2O_4$ and 0.855 gm. NaCl in 100 cc. distilled water.

3. Calcium.—Dissolve 0.40 gm. NaCl and 0.67 gm. $CaCl_2$ in 100 cc. water. This is isotonic with 0.9 per cent NaCl.

4. Lung Extract.—In our earlier experiments we prepared a saline extract of perfused dog lung. This preparation has advantages in some experiments where a high degree of purity is required. There is considerable variability in the potency of different preparations of this type, however. We have recently found that extracts of thoroughly washed but unperfused beef lung are more uniform and seem to be more stable. Since they are used in high dilution the traces of prothrombin and antithrombin present are not significant in our standard titration procedure. To make such an extract we add 100 cc. saline to 100 gm. ground beef lung. Extract at 5°C. for 24–48 hours, with frequent stirring. Strain and centrifugalize. The extract is sealed in 8 mm. glass ampules and stored until needed in vacuum bottles containing solid carbon dioxide ("dry ice"). Prior to use in titration the pH of the extract is adjusted to 7.4 with the aid of N/10 NaOH, using phenol red as an indicator. The extract is then diluted with saline to a point at which optimum results are obtained in the titration of normal control plasma. Usually the original extract is diluted about 16-fold to secure this result.

5. Oxalated Plasma.—Venous dog blood is drawn into vaselined syringes and mixed with about one-seventh its volume of isotonic potassium oxalate (1.85 per cent). Centrifugalize and note hematocrit readings in order to correct later for the oxalate dilution factor.

6. Fibrinogen.—It is made from a prothrombin-free plasma, obtained by use of $Mg(OH)_2$ as an adsorbing agent. The $Mg(OH)_2$ suspension is prepared as follows: Slowly add 25 cc. concentrated NH_4OH to 100 cc. of 20 per cent $MgCl_2$; decant and wash precipitate several times with water; centrifugalize and suspend packed precipitate in 30 cc. saline. 15 cc. of this $Mg(OH)_2$ suspension are mixed with 150 cc. oxalated plasma. After removal of the $Mg(OH)_2$ by thorough centrifugalization, the pH of the "adsorbed plasma" is adjusted to 7.4. To prepare the fibrinogen, 150 cc. of this adsorbed plasma are mixed with 50 cc. saturated $(NH_4)_2SO_4$ and centrifugalized. The precipitate is dissolved in 150 cc. oxalated saline, and the precipitation is repeated. As some fibrinogen is lost by this process, we dissolve the final precipitate in a reduced volume of oxalated saline (50 cc.). The solution is then dialyzed against several changes of oxalated saline for a total of 90 minutes at 5°C. to remove the $(NH_4)_2SO_4$. After adjusting the pH to 7.4, the fibrinogen is stored in sealed ampules in dry ice. The product keeps well for 1–2 weeks. As a dialyzing membrane we use Visking casing, manufactured by the Visking Corporation, Chicago.

7. Thrombin.—It is prepared by a modification of the method of Mills and Ling (6). To 100 cc. oxalated plasma add 100 cc. saturated $(NH_4)_2SO_4$. Centrifugalize and extract the precipitate with 100 cc. of 17.37 per cent $(NH_4)_2SO_4$. Most of the fibrinogen and some of the globulin remains undissolved. Centrifugalize. To the supernatant fluid add 4.63 gm. $(NH_4)_2SO_4$. The precipitate which now appears contains a trace of fibrinogen along with considerable globulin and most of the prothrombin. Centrifugalize and treat the precipitate with 17.37 per cent $(NH_4)_2SO_4$. Most of the precipitate dissolves. Centrifugalize. Add 4.63 gm. $(NH_4)_2SO_4$ to the supernatant fluid to bring down the prothrombin fraction for a third time. Dissolve this precipitate in 15 cc. oxalated saline. Dialyze 90 minutes at 5°C. against several changes of oxalated saline. The prothrombin is now converted to thrombin by the addition of 2 cc. of 1.2 per cent $CaCl_2$ and 1 cc. of undiluted lung extract. After 15 minutes incubation at room temperature the thrombin is dialyzed 3 hours at 5°C. against saline to remove the excess of calcium. The pH of the thrombin is adjusted to 7.4 and the solution is sealed in small glass ampules and stored in dry ice. It keeps well for several weeks.

Two modifications have been made in the actual prothrombin titration procedure. Previously the plasma was defibrinated by adding freshly "activated" serum. We now use the thrombin preparation just described. To 30 drops of plasma are added 3 drops of thrombin so diluted that a clot forms in 15–18 seconds. The second change is the use of acacia. We found that variations in the reactivity of the reagents were decreased if a small amount of acacia was present during the clotting process. We now include 1 drop of neutral 30 per cent acacia solution in the final incubation mixture. The acacia concentration in the clotting tubes is thus 2 per cent.

The present technic involves considerable improvement in the reactivity and stability of our reagents. As a result we now obtain higher prothrombin unitage than in our earlier experiments. With normal dog plasma, values of 325–400 are now obtained, in contrast to our earlier values of 200–275. As in our earlier experiments we make it a practice to run pooled plasma from several normal dogs as a control along with each unknown plasma. Instead of expressing results in prothrombin units one may express each unknown in percentage of the normal pooled control.

EXPERIMENTAL OBSERVATIONS

Prolonged chloroform anesthesia was used for the production of severe liver injury. In such animals the maximum effect, as shown by Whipple and Hurwitz (2), is seen 24–48 hours later. At this time the dog refuses to eat and is jaundiced. Bleeding occurs from needle puncture incisions, and at times from mucous membranes. A typical experiment, dog 1, is given below.

Dog 1, an 18.2 kilo male mongrel, was deprived of food for 48 hours and then given deep chloroform anesthesia for 1¾ hours. On the 2nd and 3rd days the dog was listless and refused to eat. The plasma first exhibited a trace of icterus 20 hours after anesthesia. This deepened rapidly, being most marked in the 47 hour blood sample. Most of this jaundice had disappeared by the 110th hour. From this time on the dog rapidly improved clinically, and thereafter it ate normally. On the 6th day it appeared entirely normal.

Between the 40th and 110th hours after anesthesia there was prolonged bleeding from venipuncture incisions. The clotting time was over 15 minutes and the clots which formed were flabby. The bleeding time was over 10 minutes, and the incisions frequently began to bleed again after having stopped temporarily.

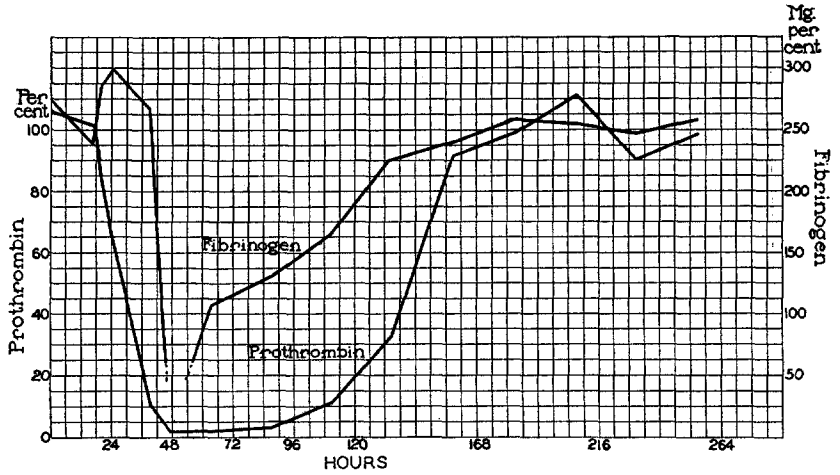

CHART 1

Chart 1 shows the fibrinogen and prothrombin curves of this dog. The fall in fibrinogen was marked, the lowest level occurring at the end of the 2nd day. At this time the clots were so flimsy that accurate estimation of the fibrinogen was no longer possible. Even more striking was the fall in prothrombin. The latter was almost completely absent at the end of 48 hours. The fall in prothrombin was not only more marked but it occurred somewhat earlier than the fall in fibrinogen. This would indicate that it is somewhat more easily depressed as the result of liver injury.

The recovery in fibrinogen began promptly on the 3rd day, and was complete by the 8th day. This coincides with the liver repair, which

begins in about 48 hours and is almost complete in 5–6 days. It is to be noted from the chart that the prothrombin returned to normal more slowly than did the fibrinogen. This again would suggest that the prothrombin level is the more delicate indicator of disturbed liver function.

The liver injury is much less severe if the anesthesia is light or of shorter duration. In such experiments intoxication is only moderate. The animal eats poorly and is slightly jaundiced. As a rule, these animals do not bleed spontaneously. Dog 2 illustrates an experiment of this type.

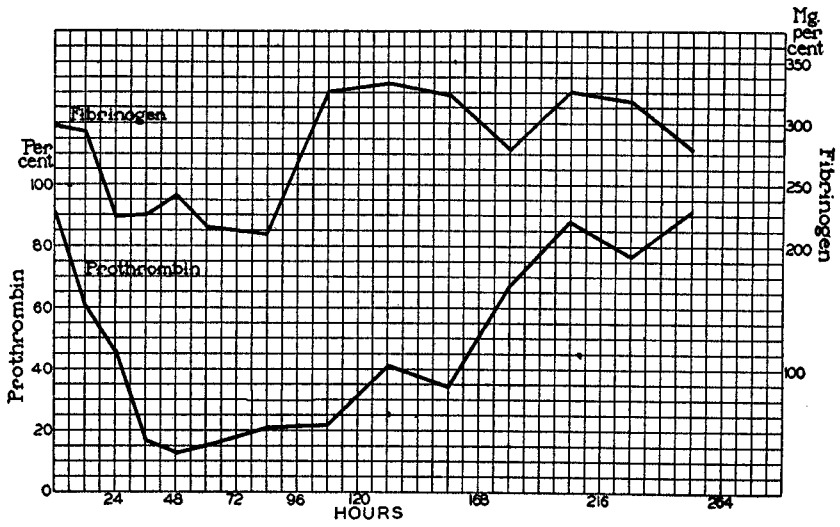

CHART 2

Dog 2, a 9.1 kilo mongrel female, was deprived of food for 48 hours and then given moderately deep chloroform anesthesia for 90 minutes. From the 2nd to the 5th day after anesthesia the plasma was slightly jaundiced, and the dog ate poorly during this period. After the 5th day, the clinical condition was excellent.

The clotting time and bleeding time were within normal limits except at the 36th hour; then the clotting time was 13 minutes and the bleeding time was 4 minutes. However, there was no spontaneous bleeding.

Chart 2 shows the fibrinogen and prothrombin curves of this dog. The prothrombin fell to a level of 14 per cent at the end of the 48th

hour after anesthesia. At this time the fibrinogen had fallen only moderately, and at its lowest point it was approximately 70 per cent of the control level. The recovery period was slightly over a week in the case of prothrombin. In contrast, the fibrinogen had returned to normal within 2 days. This shows the greater ease with which the fibrinogen level is maintained.

A rather mild but chronic type of liver injury can be produced by giving small daily doses of chloroform over a long period of time. The animals remain in fairly good condition as a rule for the first 2–4 weeks, but later they usually eat poorly and lose considerable weight. The animals gradually develop an intense jaundice which clears up slowly after the chloroform is discontinued. A bleeding tendency frequently occurs in the course of such experiments. Histologic examination of the liver at this time reveals a marked fatty metamorphosis with a few necrotic liver cells involving the central half or two-thirds of the liver lobules. Dog 3 shows a typical experiment of this type.

Dog 3, a 15 kilo mongrel female, was given chloroform daily for a period of 60 days, as indicated on Chart 3. The chloroform, mixed with 8–12 cc. cottonseed oil, was given by stomach tube. The dog was fed mixed table scraps. Jaundice, which was noted first on the 6th day, gradually increased during the first 4 weeks. On the 28th day the icterus index was 42. Thereafter there was some fluctuation in the intensity of the jaundice, with an icterus index usually between 20 and 30. During the last 10 days of the experiment the icterus index averaged 46. The jaundice had disappeared completely 3 weeks after the chloroform was discontinued. For the first 4 weeks the dog ate fairly well; the weight was 14 kilos at the end of this time. However, food was taken rather poorly during the last 4 weeks, and the weight at the end of this period was 10 kilos. After the last dose of chloroform was given, the dog improved steadily, and 3 weeks later appeared entirely normal.

Between the 20th and the 30th day, bright red blood was present in the stools on a number of occasions, and at times hematomas formed in the neck following venipunctures. The clotting time and bleeding time were greatly prolonged. On the 28th day the blood clotted only after 17 minutes; however, the clot which finally formed was solid. The bleeding time was 7½ minutes.

Chart 3 shows the fibrinogen and prothrombin curves of this dog. The amount of chloroform given was so small that the fibrinogen remained within the limits of normal throughout. The prothrombin,

however, was reduced gradually to very low levels. It was at the 10 per cent level several times during the 4th week, and at this time a bleeding tendency was present. No further spontaneous bleeding occurred during the last half of this experiment, when the prothrombin remained above the 25 per cent level. After the chloroform was discontinued, the prothrombin was restored gradually to normal levels.

The prothrombin and fibrinogen were completely dissociated in this experiment, and a bleeding tendency developed as a result of the low

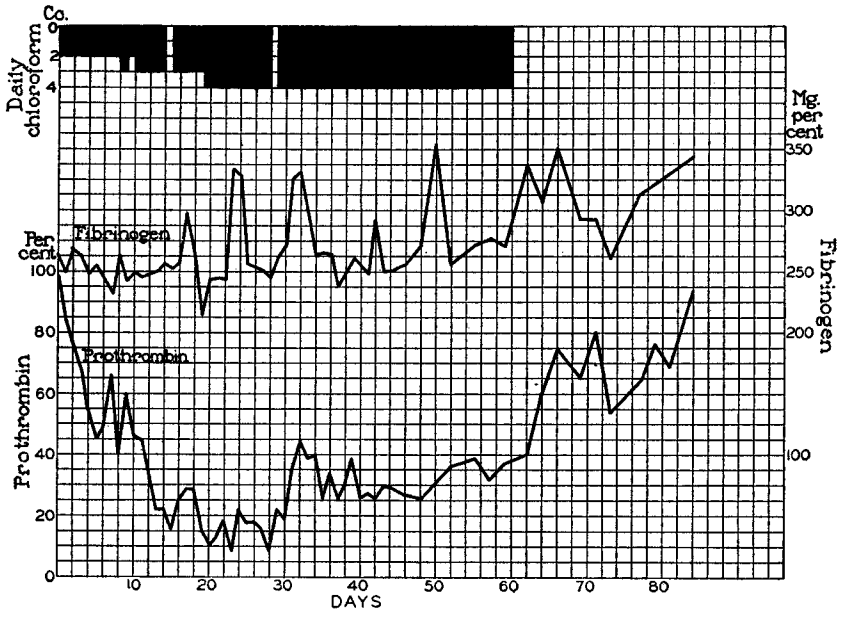

CHART 3

plasma prothrombin. It is apparent that the prothrombin falls with a mild liver injury which is not sufficiently severe to affect the more stable fibrinogen.

Another case of chronic chloroform intoxication, complicated, however, by distemper, is given below.

Dog 4, an 8.7 kilo mongrel female, was given chloroform by stomach tube over a period of 21 days, as shown in Chart 4. The first sign of distemper appeared on the 21st day. It gradually increased in severity, with death of the

animal on the 32nd day. A trace of jaundice was first noted on the 2nd day; this gradually increased, with an icterus index of 50–60 during the last week in which chloroform was given. After discontinuance of the chloroform, the jaundice decreased rapidly, until at the time of the dog's death, the icterus index was 8. Between the 13th and the 26th day the bleeding time and clotting time were prolonged. The maximum was on the 20th day, when the bleeding time was 5 minutes, the clotting time 17 minutes. All clots were very solid. Tarry stools were present on the 18th, 19th, and 20th days. Essential autopsy findings were an interstitial pneumonia, a small shallow gastric ulcer in the pyloric region, and degenerative changes in the liver. In the central one-fourth of the liver lobules

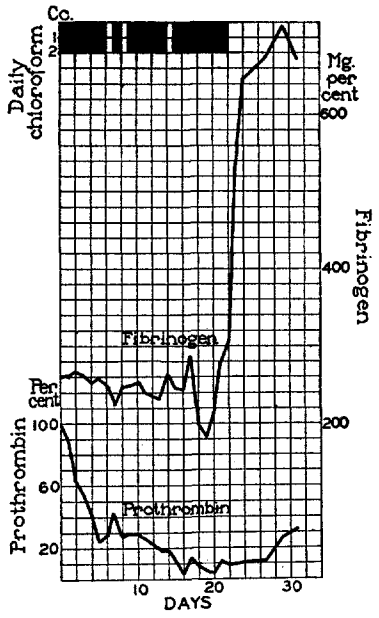

CHART 4

there was marked fatty metamorphosis with an occasional necrotic epithelial cell. Considerable regenerative activity of the surrounding liver cells was present. Bleeding from the gastric ulcer was probably responsible for the tarry stools.

This dog (see Chart 4) shows even more strikingly the dissociation of prothrombin and fibrinogen with very chronic intoxication. Here again the dosage was such that the prothrombin was chronically depressed while the fibrinogen remained within normal limits. The animal then suffered an attack of canine distemper. At this point

the fibrinogen showed the usual response to infection. Within the very brief interval of 2 days the plasma fibrinogen rose to almost twice its normal level. At autopsy the liver still showed evidence of the injury produced by chloroform. This injury prevents normal regeneration of prothrombin, but it does not interfere at all with the emergency production of large quantities of fibrinogen.

DISCUSSION

The bleeding in very acute severe chloroform intoxication is due to a deficiency in two essential clotting factors, fibrinogen and prothrombin. However, with very mild chronic intoxication there is a deficiency in only one of these factors, prothrombin, yet a bleeding tendency occurs. In several experiments of this type, platelets, antithrombin, and calcium studies showed normal or nearly normal values. This shows in a striking way that prothrombin deficiency can give rise to a bleeding tendency. Another example of this is shown in the recent experiments of Hawkins and Brinkhous (7). They found the bleeding tendency of bile fistula dogs to be due to a prothrombin deficiency. Similarly, the bleeding tendency in a case of hemorrhagic disease of the new-born which we studied recently (8) was associated with a very low prothrombin. Also, we have found greatly lowered prothrombin levels in cases of obstructive jaundice with a bleeding tendency (unpublished data). Quick, Stanley-Brown, and Bancroft (9) had previously obtained some data which suggested this possibility. A prothrombin deficiency as the essential cause of bleeding has also been reported in "sweet clover disease" (10, 11) and in "hemorrhagic chick disease" (12, 11).

Experiments which demonstrated the correlation between extent of liver damage and decrease in fibrinogen in chloroform and phosphorus intoxication at once suggested that fibrinogen is formed in the liver (2, 3). This idea was borne out by the experiments of Drury and McMaster (13) and Jones and Smith (5), in which animals, after hepatectomy, were found to be unable to regenerate fibrinogen. It appears probable from our experiments that prothrombin production is likewise dependent upon the liver. The decrease in plasma prothrombin in a given liver injury is always more striking than the lowering of the fibrinogen. In fact, the fibrinogen may even remain within

normal limits. Furthermore, with recovery of the liver, the prothrombin returns to normal more slowly than does the fibrinogen. This indicates that the prothrombin provides a more delicate test of liver function. It might be objected that prothrombin is manufactured more slowly, even by the normal liver, and for this reason is restored to normal more slowly. That this is not the case is shown by the rapidity with which prothrombin is restored in plasmapheresis experiments. In unpublished experiments we have replaced as much as 60 per cent of the animal's blood by washed red cells suspended in Locke's solution. Under these circumstances the plasma prothrombin is replaced with about the same rapidity as is the fibrinogen. The evidence available indicates that the slow restoration of prothrombin following chloroform intoxication is due to impaired liver function.

SUMMARY

The bleeding tendency in acute chloroform intoxication is due to deficiency in both plasma fibrinogen and plasma prothrombin. If the disorder is mild, no bleeding occurs. However, the prothrombin falls to rather low levels, although the fibrinogen falls only moderately.

A bleeding tendency may also be produced by giving small repeated doses of chloroform. In such experiments, the hemorrhagic tendency is due to a deficiency in prothrombin alone. The fibrinogen level is unaffected.

The relation of the liver injury to the plasma prothrombin level indicates that the liver is concerned in the manufacture of prothrombin. Prothrombin formation appears to be more easily interfered with than does fibrinogen formation.

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