# LIVER INJURY, LIVER PROTECTION, AND SULFUR METABOLISM

## Methionine Protects against Chloroform Liver Injury Even When Given after Anesthesia*

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The experiments listed below may seem unrelated, but, in fact, bear on the question of the defense mechanism of the liver cells against chloroform injury. It came as a complete surprise to us when experiments showed that methionine gave considerable protection against liver injury even when given 3 to 4 hours *after the anesthesia.*

The observed fact that the livers of fetuses or of newborn pups showed unexpected tolerance to chloroform injury has long interested us. The newborn pups exhibit remarkable tolerance to chloroform injury and this peculiar quality lasts 2 to 3 weeks after birth, as shown years ago by one of us (20). Tables 3 and 2 show that the nitrogen sulfur ratio of pups and normal adults is about the same, that is, 11 or 12 to 1. The protein-depleted dog, which is so susceptible to liver injury, shows a nitrogen sulfur ratio of 14 or 15 to 1, which means presumably less available sulfur-containing amino acids.

It is obvious, too, that during long protein depletion (4 to 8 weeks) on a very low protein diet, the liver is drained of its sulfur a little more completely than of its nitrogen. This sulfur deficit in the liver can be made up very promptly by feeding methionine and less effectively by cystine.

Previous reports (13, 14) from this laboratory have shown that the protein-depleted dog is extremely susceptible to chloroform liver injury, and that a single large protein feeding protects the protein-depleted dog from an otherwise fatal chloroform anesthesia and related liver injury. It was then shown (14) that *dl*-methionine and, to a less extent, *l*-cystine, given before chloroform anesthesia, are as effective in protecting the protein-depleted dog as a large protein feeding. A variety of non-sulfur-containing amino acids were found to be entirely lacking in this protective action.

### *Methods*

*Chloroform Anesthesia Experiments.*—All dogs used in these experiments were active healthy adults. As indicated in detail in the individual protocols, the dogs' reserve stores of protein were depleted by maintenance on a *very low protein diet* con-

* We are indebted to Eli Lilly and Company for aid in conducting this work.

sisting of sucrose 72.2 per cent, salt mixture (19) 4.6 per cent, bone ash (largely calcium phosphate) 4.6 per cent, crisco 14.9 per cent, mazola oil 6.5 per cent, cod liver oil 1.4 per cent, yeast powder (Fleischmann's Type 200-B), 0.7 per cent, powdered liver extract (Lilly, H8083), 0.7 per cent, nicotinic acid 13.9 mg. per cent, choline chloride 111 mg. per cent. The amount of protein in this diet did not exceed 1½ gm. per 100 gm. as fed.

The amounts of *dl*-methionine (Merck and Co.),[1] *l*-cystine (Eastman Kodak), and choline chloride (Eastman Kodak), and the routes of administration are given in the individual protocols. Other methods are described in previous reports (13, 14).

*Analysis of Livers.*—In all cases the livers were rapidly removed, and the excess blood or perfusion fluid removed by gently blotting with filter paper. The livers were then cut into small pieces, weighed, and dried to constant weight at 76°C. (usually about 72 hours). The dried liver was then ground to a powder and samples taken for analysis.

The total nitrogen determinations on liver tissue were done by macro-Kjeldahl, and the total sulfur by the wet ashing of Masters (11), using concentrated nitric and perchloric acids, followed by precipitation of sulfate as barium sulfate.

## EXPERIMENTAL OBSERVATIONS

In experiments previously reported it was shown that as little as 3.0 gm. of *dl*-methionine given *before* anesthesia would protect completely the protein-depleted dog from the effects of a 40 minute chloroform anesthesia. This was in distinct contrast to the almost invariably fatal liver damage following only a 20 minute anesthesia in the unprotected protein-depleted dog.

Experiments 1 and 2 show a totally unexpected response. Methionine given intravenously *3 and 4 hours after the start of a 30 minute chloroform anesthesia prevents the fatal injury*, but does not prevent some liver damage. On the basis of clinical condition, icterus index, and blood fibrinogen levels, dog 40-251 which received methionine 4 hours after chloroform anesthesia presented the picture of the more severe liver injury.

In Experiment 3, dog 40-251 was moderately protected by cysteine plus choline given intravenously 3 hours *after* chloroform anesthesia. It is interesting to note that the blood fibrinogen level did not fall below 250 mg. per cent despite the apparently severe liver damage. This is probably related to the presence of several purulent ulcers of the skin, as it is well known that suppuration raises the level of blood fibrinogen, even in the presence of chloroform liver injury.

[1] We are indebted to Merck and Co. for valuable amino acids.

TABLE 1

*Methionine and Cysteine Given 3 to 4 Hours after Chloroform Protects Liver*

*Experiment 1 (dog 39-230). Methionine gives moderate protection*

| Time before and after chloroform | Fibrinogen | Icterus index | Clinical condition |
|---|---|---|---|
| *hrs.* | *mg. per cent* | | |
| 0 | 420 | 0 | Normal (plasma protein = 5.10 gm. per cent) |
| 30 min. chloroform anesthesia, *dl*-methionine given by vein 3 and 6 hrs. after start of anesthesia (total 3 gm.) | | | |
| 6 | 390 | 0 | Normal |
| 24 | 280 | 2 | Good, vomited some mucus |
| 48 | 130 | 9 | Excellent |
| 72 | 140 | 13 | " |
| 96 | 187 | 11 | " |
| 120 | 240 | 6 | " recovery |
| *Experiment 2 (dog 40-251). Methionine gives moderate protection* | | | |
| 0 | 315 | 0 | Normal (plasma protein = 5.06 gm. per cent) |
| 30 min. chloroform anesthesia, *dl*-methionine given by vein 4 hrs. after start of anesthesia (total 3.0 gm.) | | | |
| 24 | 210 | 8 | Somewhat quiet, but good |
| 48 | 114 | 11 | Good |
| 72 | 102 | 17 | " |
| 96 | — | 14 | " |
| 216 | 282 | 3 | Excellent recovery |
| *Experiment 3* (dog 40-251). Cysteine + choline gives moderate protection* | | | |
| 0 | 260 | 0 | Normal (plasma protein = 5.22 gm. per cent) |
| 30 min. chloroform anesthesia, cysteine (2.85 gm. of hydrochloride) + choline hydrochloride (1.0 gm.) given by vein 3 hrs. after anesthesia | | | |
| 24 | 250 | 13 | Plasma protein 5.06 gm. per cent<br>Intoxicated, vomited mucus, weak |
| 48 | 250 | 16 | Very weak, vomited mucus. Skin ulcers |
| 72 | 340 | 14 | Improved. Ulcers purulent |
| 96 | 410 | 14 | Good recovery |

* Interval of 18 wks. between Experiment 2 and Experiment 3.

## *Clinical Histories*

*Experiment 1*, dog 39-230. Mongrel hound. Feb. 22, 1941—Weight 9.1 kg. Plasma protein level 6.32 gm. per cent. Placed on low protein diet, 135 gm. daily.

Feb. 23 to Apr. 29—Ate an average of 60 to 80 per cent of food. Apr. 30—Fasted. May 1—Weight 8.5 kg. Plasma protein level 5.10 gm. per cent. *Chloroform* anesthesia —See Table 1. The *dl*-methionine in this and the following experiments was dissolved in about 150 cc. of physiological saline and given intravenously. May 1 to May 6—Ate an average of 10 per cent of diet. May 7—Placed on kennel diet. Weight 8.0 kg.

*Experiment 2*, dog 40-251. Mongrel hound, male. May 28 to June 9, 1941—Fasted. Plasma protein level on June 5, 6.68 gm. per cent, weight 12.2 kg. June 10 to Aug. 4—Low protein diet, 200 gm. daily, eaten 95 to 100 per cent. Plasma protein level, July 29, 4.85 gm. per cent. Aug. 5—Fasted. Aug. 6—Weight 10.3 kg. Plasma protein level 5.06 gm. per cent. *Chloroform* anesthesia—See Table 1. Methionine given 4 hours after chloroform. Aug. 6 to 15—Ate 95 to 100 per cent of diet except on Aug. 7, when only 40 per cent was eaten.

*Experiment 3*, dog 40-251. Nov. 12 to Dec. 13, 1941—On low protein diet, 200 gm. daily, eaten 100 per cent. Weight on Nov. 14, 11.0 kg. Dec. 14—Fasted. Noted a number of small ulcerated areas on skin pressure points. Dec. 15—Weight 9.7 kg. Plasma protein level 5.22 gm. per cent. *Chloroform* anesthesia—See Table 1 (choline chloride and cysteine hydrochloride neutralized with sodium bicarbonate before being given intravenously; 0.6 mg. atropine sulfate intramuscularly to control salivation). Dec. 15 to Dec. 19—Ate practically no food. Ulcerated areas frankly purulent. Dec. 20—Returned to kennels.

Experiments 4 and 5 show that *dl*-methionine, given intravenously as in the above experiments 4 and 6 hours respectively *after the start of the anesthesia*, fails to prevent fatal liver damage with typical severe hyaline central liver necrosis. Evidently 4 hours is about the time limit beyond which methionine ceases to protect the liver against chloroform injury due to 30 minutes of anesthesia.

Experiment 6 confirms previous observations (6), that relatively large amounts of choline given *before* anesthesia fail to prevent fatal liver damage.

TABLE 1-*a*

*Methionine Given 4 to 6 Hours after Chloroform and Choline Given before Chloroform Fail to Protect Liver*

| Time before and after chloroform | Fibrinogen | Icterus index | Clinical condition |
|---|---|---|---|
| *hrs.* | *mg. per cent* | | |
| *Experiment 4 (dog 40-404). Methionine fails to protect when given 4 hrs. after anesthesia* | | | |
| 0 | 266 | 0 | Normal (plasma protein = 4.99 gm. per cent) |
| 30 min. chloroform anesthesia, methionine given by vein 4 hrs. after start of anesthesia (total 3.0 gm.) | | | |
| 24 | 100±* | 20 | Droopy |
| 40‡ | | | Dead. Liver, extreme hyaline necrosis |
| *Experiment 5§ (dog 30-230). Methionine fails to protect when given 6 hrs. after anesthesia* | | | |
| 0 | 387 | 0 | Normal (plasma protein = 4.45 gm. per cent) |
| 30 min. chloroform anesthesia, methionine given by vein 6 and 20 hrs. after start of anesthesia (total 3.2 gm.) | | | |
| 24 | 239 | 20 | Fair. Vomited mucus |
| 36‡ | | | Dead. Liver, extreme hyaline necrosis |
| *Experiment 6 (dog 39-338). Choline before anesthesia fails to protect* | | | |
| 0 | 355 | 0 | Normal (plasma protein = 5.07 gm. per cent) |
| 20 min. chloroform anesthesia, choline hydrochloride (2.0 gm.) by mouth 3½ hrs. before anesthesia | | | |
| 24 | 209 | 13 | Severely intoxicated, vomited mucus |
| 34‡ | | | Dead. Liver, extreme hyaline necrosis |

* Poor clot in fibrin formation with calcium chloride.

‡ Estimated hour of death.

§ Interval of 13 wks. between Experiment 1 and Experiment 5.

## *Clinical Histories*

*Experiment 4*, dog 40-404. Mongrel hound, male. July 11, 1941—Weight 11.8 kg. Started on low protein diet, 200 gm. daily, following 10 days' fast. July 12 to July 31—Ate 90 to 100 per cent of food. Aug. 1—Ate 30 per cent of food. Plasma protein level 5.03 gm. per cent. Aug. 2 to Aug. 6—Added 5 gm. fresh pig liver to daily diet. Ate 90 to 100 per cent of food. Aug. 7—Fasted. Aug. 8—Weight 10.2 kg. Plasma protein level 4.99 gm. per cent. *Chloroform* anesthesia 30 minutes' duration started 4 hours before methionine was given—See Table 1-*a*. Aug. 10—Found dead. In the gross and microscopically the liver showed severe central necrosis.

*Experiment 5*, dog 39-230. June 5—Returned to low protein diet following 10 days' fast. Plasma protein level 5.45 gm. per cent. Weight 7.3 kg. June 6 to July 28—Ate an average of 35 to 40 per cent of diet. July 29 to Aug. 4—Added 10 gm. of fresh pig liver to daily diet. Ate an average of 80 per cent of food. Aug. 4—Fasted. Aug. 5—Weight 5.8 kg. Plasma protein level 4.45 gm. per cent. *Chloroform* anesthesia—See Table 1-*a*. Aug. 7—Found dead. Autopsy reveals unclotted blood in great vessels and heart. In the gross and microscopically the liver showed severe central necrosis.

*Experiment 6*, (dog 39-338). Mongrel hound, male. Oct. 1 to Dec. 20, 1941—Low protein diet 185 gm. daily. Ate average of 95 per cent of food. Dec. 20 to Dec. 24—Ate 60 to 70 per cent of food. Dec. 24 to Jan. 3, 1942—Added 10 gm. fresh pig liver to daily diet. Ate 95 per cent of food. Jan. 4—Ate 60 per cent of food. Weight 11.1 kg. Jan. 5—Ate 50 per cent of food; choline chloride added to give 0.60 gm. total ingested. Jan. 6—Ate about 50 per cent of food; choline chloride added to give 1.0 gm. of choline chloride ingested. Jan. 7—Fasted. Jan. 8—Plasma protein level 5.07 gm. per cent. Gave 2.0 gm. of choline chloride by stomach tube, preceded by 0.6 mg. of atropine sulfate intramuscularly to prevent excess salivation. *Chloroform* anesthesia 20 minutes started 3½ hours after choline was given. See Table 1-*a*. Jan. 10—Found dead. Autopsy reveals severe central necrosis of liver both in the gross and microscopically.

*Histological Specimens* (Table 1-a)

Dog. 39-404—Typical extreme hyaline central liver necrosis; a few liver cells about the portal structures are not necrotic. Dogs 39-230 and 39-338 present an identical picture.

In Table 2 are listed the nitrogen and sulfur analyses of *adult dog livers*. It is obvious that the liver N/S ratio in the *dog well fed with protein* is distinctly lower (10.5 to 12.4) than the liver N/S ratio in the *protein-depleted dog* (14.1 to 15.4). The feeding of *l*-cystine (dog 39-215) or *dl*-methionine (dog 39-164) lowered the *liver* N/S ratio markedly in both cases. The response to methionine feeding is greater though less total methionine was fed. The comparatively small change in the muscle N/S ratio makes it appear that the liver can hoard the S-containing amino acids in some form. It is not unlikely that much of the methionine is incorporated in the proteins of the liver cell.

In the adult dogs the milligrams of liver N per cent body weight in the protein-depleted dogs do not vary significantly from those of the normal well fed dogs. This may be attributed to the fact that, with long extended protein deprivation, the percentage decrease in total carcass protein probably reaches about the same value as the liver protein decrease. In rats, Addis, Poo, and Lew (2) have demonstrated essentially this fact.

TABLE 2

*Liver Nitrogen and Sulfur*

*Normal Well Fed Adult Dogs*

| Dog No. | Plasma protein level | Body weight | Liver weight | Liver solids | Dry liver | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | N | S | $\frac{N}{S}$ | Mg. N | Mg. S |
| | | | | | | | | per cent body weight | |
| | *gm. per cent* | *kg.* | *gm.* | *per cent* | *per cent* | *per cent* | | | |
| 5E | | 18.6 | 539 | 26.9 | 9.21 | 0.752 | 12.2 | 72 | 5.9 |
| 2F | | | 306 | 24.8 | 10.64 | 0.885 | 12.0 | | |
| 1F | | | 320 | 23.4 | 10.58 | 0.860 | 12.3 | | |
| 40-167 | | 14.8 | 377 | 19.2 | 11.65 | 0.941 | 12.4 | 57 | 4.6 |
| 41-137 | 6.44 | 15.4 | 435 | 19.0 | 10.27 | 0.942 | 10.9 | 55 | 5.0 |
| 41-127 | 6.19 | 12.6 | 355 | 27.4 | 9.54 | 0.896 | 10.6 | 74 | 7.0 |
| 41-138 | 6.58 (5.21) | 14.0 | 362 | 26.2 | 10.42 | 0.858 | 12.2 | 70 | 5.7 |
| 41-130 | 6.08 (4.80) | 11.7 | 315 | 26.2 | 10.94 | 1.03 | 10.5 | 77 | 7.3 |
| Average..... | | | | 24.1 | 10.41 | 0.896 | 11.6 | | |
| *Protein-Depleted Adult Dogs* | | | | | | | | | |
| 39-10* | 4.90 | 8.2 | 214 | 27.1 | 7.58 | 0.527 | 14.4 | 54 | 3.8 |
| 35-6 | 4.14 | 18.0 | 790 | 26.2 | 7.17 | 0.502 | 14.3 | 82 | 5.7 |
| 39-230 | 4.45 | 7.7 | 230 | 22.5 | 8.91 | 0.631 | 14.1 | 81 | 5.7 |
| 41-305 | | | 313 | 26.6 | 11.23 | 0.736 | 15.3 | Pregnant | |
| 41-308 | | | 377 | 33.0 | 7.02 | 0.454 | 15.4 | " | |
| Average..... | | | | 27.1 | 8.38 | 0.570 | 14.7 | | |
| *Protein-Depleted Adult Dogs Fed Methionine or Cystine* | | | | | | | | | |
| 39-164‡ | 4.00 | 7.4 | 238 | 27.8 | 5.65 | 0.674 | 8.4 | 51 | 6.1 |
| 39-215§ | 4.95 | 12.1 | 597 | 31.2 | 5.60 | 0.494 | 11.3 | 85 | 7.5 |

* Muscle $\frac{N}{S} = \frac{12.79}{0.72} = 17.8$.

‡ Muscle $\frac{N}{S} = \frac{11.40}{0.75} = 15.2$; *dl*-methionine, 1.0, 1.0, 1.5 gm. fed on 3 days respectively before sacrifice.

§ Muscle $\frac{N}{S} = \frac{13.10}{0.83} = 15.8$; *l*-cystine, 1.0, 1.0, 1.0, 1.5 gm. fed on 4 days respectively before sacrifice.

*Histological Specimens* (Table 2)

Dog 41-137—Cells are normal; glycogen granules are obvious.

Dog 41-127—Cells are normal; glycogen granules are conspicuous.

Dogs 41-138 and 41-130—Cells are normal.

Dog 39-10—Liver cells are normal. Glycogen is conspicuous in liver cells in central half of lobule.

Dog 35-6—Liver cells are normal, stuffed with glycogen and have the appearance of vegetable cells.

Dog 39-230—Liver shows usual extreme liver necrosis.

Dog 41-305—Fatal chloroform poisoning—usual extensive hyaline liver necrosis (pregnancy).

Dog 41-308—Fatal chloroform poisoning and liver injury (pregnancy).

Dog 39-164—Liver cells are stuffed with glycogen (like 35-6).

Dog 39-215—Liver cells are stuffed with glycogen (like 35-6).

Table 2 contains several individual experiments which deserve comment.

The first 4 dogs (5E, 2F, 1F, and 40-167) had been used for radio iron metabolism experiments, were on a high protein intake, and were all young, active, and healthy adults. They were then perfused under ether anesthesia. Dogs 41-137 and 41-127 were normal dogs on a mixed diet, fasted 24 hours, and killed under ether after a perfusion which freed the liver tissue of blood. These dogs show a normal plasma protein level and liver tissue which is normal histologically. Although there is much difference in the amounts of liver solids, the N/S ratio is similar, and we suspect a "dilution" of the cell protein by fat or glycogen in dog 41-127.

Dogs 41-138 and 41-130 were well fed dogs which had been subjected to a rapid and severe *plasma depletion* (bleeding and return of red cells suspended in saline) which had lowered the plasma protein levels from 6.58 to 4.23 gm. per cent and from 6.08 to 3.58 gm. per cent respectively. After 24 hours the plasma protein levels had risen to 5.21 and 4.80 gm. per cent respectively, presumably due to rapid influx of plasma protein from reserve body stores (liver and muscle). The dog was then perfused under ether anesthesia. This procedure did not disturb the figures for nitrogen and sulfur in the liver.

*Protein-depleted dogs* (Table 2) show many points of clinical interest.

Dog 39-10 had been on a very low protein diet (not more than 2 to 3 gm. protein per day) for 13 weeks and was exsanguinated under ether anesthesia. The plasma protein level was low (4.90 gm. per cent) and the percentage of N and S were both much below normal, but the S was the lower, giving a high N/S ratio, 14.4. Dog 35-6 was both anemic and plasma-depleted. One week's protein fast was followed by 4 weeks of a very low protein diet (not more than 2 to 3 gm. protein per day). The dog was then perfused under ether. The plasma protein level was very low (4.14 gm. per cent), and there had been a loss of 3.5 kilos body weight. The N and S values are almost exactly those of the preceding experiment.

In dog 39-230 (refer to Table 1-*a* and Clinical history), a 10 day fast was followed

by the low protein diet for 9 weeks. Plasma protein level (4.45 gm. per cent) was low and there had been a loss of weight of 2 kilos. Chloroform anesthesia 30 minutes caused death in 36± hours. The liver showed typical extreme hyaline necrosis. The N and S analyses are much like those of plasma depletion alone. The susceptible and damaged liver cells evidently contain about the same amount of protein (Table 2). Methionine given 6 hours after chloroform was not taken up in significant amounts by these injured and necrotic liver cells—compare with dog 39-164, (Table 2) where methionine uptake is obvious.

*Pregnant dogs* 41-305 and 41-308 (Table 2) were obviously close to term. They were not well nourished and we know nothing about their dietary history. They were both fasted for 3 days. The dogs went into labor. Pups 308-1 and 2, and 305-1, 2, 3, and 4 were born (Table 3). The mothers were then given light surgical chloroform anesthesia—Dog 41-305 50 minutes and dog 41-308 70 minutes. Recovery from chloroform anesthesia was prompt and during the ensuing 4 hours pups 305-5c, 6c, and 7c, and pups 308-3c, 4c, and 5c were born normally. The mother dogs died in about 15 hours following the chloroform anesthesia with ample evidence of fatal chloroform liver injury. The pups' livers exposed to the same dose of chloroform *in utero* showed not a trace of injury (Table 3 and Histological specimens).

Protein-depleted dogs 39-164 and 39-215 (Table 2) had been on a low protein diet for 9 weeks which had effected some hypoproteinemia (4.00 and 4.95 gm. per cent respectively). They were fed methionine and cystine (Table 2) and 24 hours later were exsanguinated under ether. The content of the dry liver in N and S is decidedly low as compared with normal dogs, but the N/S ratio is decreased in comparison with protein-depleted dogs not fed the S-containing amino acids. Obviously the liver tissue has taken up these amino acids more than the muscle tissue and more of the methionine than of the cystine. The increase in liver solids is certainly in large part due to glycogen (Histological specimen). The low content of nitrogen *might* be due to metabolic activity related to the methionine and cystine intake—a turn over of protein to supply other body protein emergency needs. The S is largely retained in the body in such experiments (17).

TABLE 3

*Liver Nitrogen and Sulfur*

*Normal Pups (48 Hrs. Old)*

| Pup No. | Weight | Liver weight | Liver solids | Dry liver | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | N | S | $\frac{N}{S}$ | Mg. N | Mg. S |
| | | | | | | | per cent body weight | |
| | *kg.* | *gm.* | *per cent* | *per cent* | *per cent* | | | |
| 1 | 0.32 | 12.3 | 22.2 | 12.77 | 0.949 | 13.4 | 109 | 8.1 |
| 2 | 0.33 | 13.0 | 23.5 | 11.30 | 0.892 | 12.8 | 112 | 8.8 |
| 3 | 0.31 | 11.6 | 22.9 | 12.78 | 0.988 | 12.9 | 110 | 8.5 |
| 4 | 0.33 | 13.0 | 23.8 | 13.59 | 1.17 | 11.8 | 127 | 10.8 |
| Average..... | | | 23.1 | 12.61 | 0.998 | 12.7 | | |
| *Pups Born before Chloroform Anesthesia (7 Hrs. Old)* | | | | | | | | |
| 308-1 | 0.25 | 12.0 | 30.7 | 7.87 | 0.720 | 10.9 | 116 | 10.6 |
| 308-2 | 0.23 | 13.0 | 26.9 | 6.84 | 0.633 | 10.8 | 104 | 9.6 |
| 305-1 & 2 | 0.35 | 16.0 | 28.8 | 8.16 | 0.735 | 11.1 | 109 | 9.8 |
| 305-3 & 4 | 0.43 | 20.5 | 26.8 | 7.07 | 0.641 | 11.7 | 91 | 7.8 |
| Average..... | | | 28.3 | 7.48 | 0.682 | 11.1 | | |
| *Pups Born after Chloroform Anesthesia (48 Hrs. Old)* | | | | | | | | |
| 308-3 & 4c | 0.42 | 15.6 | 24.5 | 12.34 | 1.07 | 11.5 | 113 | 9.8 |
| 308-5c | 0.18 | 7.4 | 21.2 | 13.02 | 1.01 | 12.9 | 116 | 9.0 |
| 305-5c | 0.14 | 10.0 | 22.1 | 12.40 | 1.03 | 12.0 | 203* | 16.9 |
| 305-6 & 7c | 0.39 | 17.9 | 22.5 | 11.37 | 0.967 | 11.8 | 118 | 10.0 |
| Average..... | | | 22.6 | 12.28 | 1.02 | 12.1 | | |

* Out of cage during night—cold and dehydrated but viable.

## *Histological Specimens* (Table 3)

Pup 1—Cells are normal; marrow cells are numerous.

Pup 2—Cells are normal; marrow cells are numerous. Glycogen is well shown.

Pup 3—Cells are normal; marrow cells are numerous.

Pup 4—Cells are normal; marrow cells are numerous. A few bile canaliculi show brown colloid deposits. Lobulation is very well outlined due to contained blood.

Pups 305-1, 2, 3, and 4—Cells are normal, but stuffed with glycogen; marrow cells are numerous.

Pup 308-1—Cells are normal, but stuffed with glycogen; marrow cells are numerous.

Pup 305-5c—Cells are normal; there is no necrosis; marrow cells are numerous. A few bile canaliculi contain brown colloid.

Pup 305–6c—Cells are normal; there is no conspicuous glycogen; no bile canaliculi visible; and the marrow cells are numerous.

Pup 305-7c—Cells are normal; glycogen is visible and fairly abundant; marrow cells are numerous; and there is no necrosis.

Pup 308-3c—Cells are normal; there are no glycogen granules, no necrosis, and marrow cells are numerous.

Pups 308-4 and 5c—Cells are normal, there are no glycogen granules, no necrosis marrow cells are numerous. A few bile canaliculi contain brown colloid.

Table 3 shows the nitrogen and sulfur values for pups' livers both normal and exposed to chloroform *in utero*. It is fair to say that in the 48 hour pups, whether exposed to chloroform or not, the N and S values and N/S ratio fall within the range of normal adult dogs. In the pups 7 hours after birth the glycogen deposits are very great but the N/S ratio is unchanged. The amount of N to body weight shows that the pups have livers larger in proportion than adults. Chloroform anesthesia sufficient to kill the mother within 15 to 20 hours causes no abnormality in the pups *in utero* during the anesthesia recognizable by chemical analysis (Table 3) or by histological study.

## DISCUSSION

What mechanism accounts for the liver cell necrosis due to chloroform? No adequate explanation has yet been given, but we have suggested that the —SH groups of vital enzyme systems may be concerned (14). We have also speculated on the relation between liver sulfhydryl and the tension of available oxygen; the importance of the available oxygen tension for decreasing chloroform liver damage has been emphasized by the work of Goldschmidt, Ravdin, and Lucké (5). We need not repeat that argument, but may say that the rapid uptake by the depleted liver cell of fed methionine and cystine (Table 2) supports these hypotheses.

What shall we say of the observation that methionine given 3 or 4 hours *after chloroform* anesthesia gives definite protection to the protein-depleted dog? At first sight this may seem to confuse the issue, but any adequate explanation of chloroform liver necrosis must comprehend this observed fact. We may say that the injury done the liver cell by chloroform during the anesthesia period is in a measure reversible during a 3 or 4 hour period. If a disturbance of an enzyme system is responsible, then that process is reversible during an interval of 3 or 4 hours.

When *methionine* (a single dose) is fed to a protein-depleted dog, it is retained within the body (17). It does not appear in great concentration in the muscles but does appear in large amounts in the liver (Table 2). We believe the methionine is *incorporated* in the liver protein matrix just as readily as the same protein matrix was *depleted* of its sulfur by prolonged low protein diet periods.

*Liver proteins* are in a state of constant flux as incoming materials are synthesized into protein and out going proteins (*e.g.* fibrinogen, prothrombin) are constantly supplied to fill urgent body requirements. There must be some modification of liver protein to produce plasma proteins (fibrinogen) just as plasma proteins must be modified slightly when they supply the protein needs of body cells (21). This implies that liver proteins have a variable make-up—that these proteins are a part of a dynamic system—that certain amino acids may be lost or again regained without serious disturbance of the cell function. There are opinions for and against this argument. In favor are the experiments of Schoenheimer and Rittenberg (16), Schenk and Wollschitt (15); against is the paper of Lee and Lewis (10).

*Pups* after birth or fetuses *in utero* have an extraordinary *tolerance* for chloroform anesthesia. After birth the pups slowly lose this peculiar tolerance for chloroform and by the 4th week have reached the level of the adult dog (20). Chemical analyses show no significant differences between pups and normal adults (Tables 2 and 3). It is noteworthy, however, that the N/S ratio of the pups is normal as compared with healthy adults but that the *mothers* showed a very high N/S ratio, that is, *less sulfur*. Apparently the fetuses *in utero* can rob the mother liver of sulfur, as is also true for iron. The needs of the growing fetus take precedence over those of the maternal body—a very important biological law.

One structural difference must be mentioned—the presence of blood islands or *marrow cells* within the liver lobules in the pups. These islands of marrow cells gradually disappear as the pup loses its tolerance for chloroform. This fact is readily demonstrated but adequate explanations to fit this observation are not yet at hand.

The liver lobules of the fetus or pup except for these blood-forming cells are much like the adult liver lobules. The liver lobule of the newborn pup is a little smaller than the adult liver lobule; and the cell nuclei a little larger and more active looking, and more mitoses are to be found.

The pup's liver within 9 months grows to its adult size—an increase of about 20 times. What goes on in the liver lobules meanwhile is not mentioned in the textbooks of anatomy. We get the impression that the formation of new lobules is not accepted but the biliary tree *must* lengthen as the liver size expands. The common duct at the hilum, let us say, is a fixed point and the liver capsule moves out as the liver weight increases. The biliary tree must lengthen its branches and almost certainly the terminal arborizations must increase in number or, in other words, new lobules must be formed. New liver cells must be formed rapidly during growth but these changes apparently have little effect upon chloroform tolerance. The *cross section* of the liver lobules remains relatively unchanged and therefore there is no *structural* basis for the observed tolerance of pups to anoxia.

From the work of Himwich and his collaborators (4, 9) we know that newborn pups are highly resistant to the injurious effects of anoxia. This tolerance to anoxia may be a part of the same physiological mechanism which renders these pups so resistant to chloroform anesthesia.

The presence of blood-forming cells within the liver remains as one certain structural difference between these fetuses or newborn pups and the adult dogs. However these marrow cells are not uniformly distributed in the liver lobules and one never sees occasional areas of liver cells (free of marrow cells) involved in necrosis and other areas (containing marrow cells) uninvolved in the liver injury. We have only one suggestion to make—that the presence of these marrow cells in some obscure fashion modifies the enzyme system, disturbance of which in the normal adult dog is responsible for the liver necrosis.

As noted above (Table 2) the *protein-depleted dog* is a dog even more completely depleted of sulfur. The maximum differences between the liver N/S ratio of the protein-depleted dog and the normal well fed dog seem too large to be explained solely on the basis of fluctuations in the non-protein sulfur content (*e.g.* glutathione). It appears to connote a definite loss by the protein-depleted liver of some relatively sulfur rich component, presumably protein in nature, the loss of which makes the liver more susceptible to a variety of injurious agents known and unknown. When methionine (or cystine) is fed to this type of dog there is a rapid uptake of sulfur, in the liver especially. This response makes up the liver sulfur deficit very promptly. In striking contrast (dog 29-230, Table 1-*a*; and Table 2) when the methionine is given 6 hours *after* the chloroform anesthesia there is no uptake of methionine by the liver. The liver cells are fatally damaged by the chloroform in this experiment and do not take up the methionine. Obviously this uptake of methionine is not a simple physical response (adsorption) nor is the response related to the Kupffer cells of the liver (reticulo-endothelial system) as these cells are not specifically injured by the chloroform. *Viable liver cells* are essential for the rapid uptake of methionine.

The effects of *protein depletion* on the liver are of general interest not only because of the increased susceptibility to obvious hepatoxic agents, such as chloroform or arsphenamine (12), but also because of its significant relationship to other disease states—fatty livers (1), experimental cirrhosis (3, 7), experimental liver carcinoma (8), and decreased liver function (18). In almost all of these conditions the beneficial effect of a high protein dietary or more specifically of methionine or cystine or cysteine *plus* choline, points to a fundamentally close relationship between all these abnormal states.

Liver injury has been treated in the past and still is being treated with a high carbohydrate diet. There is good reason for the carbohydrate therapy but, as a result of work in this and other laboratories demonstrating the paramount importance of protein in preventing or allaying liver damage, a diet high in

protein as well as carbohydrate obviously is the correct therapy. The protective action of methionine or cystine plus choline suggests the use of these substances or of suitable methionine-rich protein digests (*e.g.* casein) as therapeutic agents where any type of liver damage is present. Methionine in solution can be administered parenterally alone or with glucose without any unfavorable reaction in man and animal.

## SUMMARY

Protein-depleted dogs are very susceptible to injurious agents—in particular, chloroform. Methionine given shortly *before* chloroform anesthesia will give complete protection against chloroform. Methionine (or cysteine plus choline) given 3 or 4 hours *after* chloroform anesthesia will give significant protection against the liver injury of chloroform anesthesia. Methionine given more than 4 hours after chloroform anesthesia gives no protection against liver injury. Choline alone given before chloroform gives no protection against liver injury.

The protein-depleted dogs have livers which are deficient in both nitrogen and sulfur, but sulfur is depleted more than is the nitrogen. The N/S ratio therefore rises. Methionine or cystine feeding promptly makes up this liver sulfur deficit. Viable liver cells are necessary for this uptake of sulfur.

Livers of fetuses *in utero* or of newborn pups tolerate a chloroform anesthesia which will cause fatal liver injury in adults. The nitrogen and sulfur values of these fetus or pup livers are within the high normal values for adults. *Blood-forming cells* are present in the fetus or pup livers during this period. When these blood islands are eliminated during the 3rd or 4th week of life, the liver then becomes normally susceptible to chloroform liver injury.

Methionine or methionine-rich protein digests (*e.g.* casein) or various proteins by mouth or by vein should prove useful to protect the liver against certain types of injury and to aid in organ repair.

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