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(Addendum – Appendix A, 18 March 2013)

To: Michael L. Dourson, Ph.D., DABT, ATS
Toxicology Excellence for Risk Assessment (TERA)

Subj: – Review of liver slides from the National Cancer Institute's Bioassay of 1,4-Dioxane for Possible Carcinogenicityconducted in 1978[1]

Following are the results of my reanalysis of the haematoxilin and eosin (H&E) stained histopathology slides from the liver of mice from the subject study. This reanalysis was performed at the Experimental Pathology Laboratories (EPL), Research Triangle Park, NC during September through November 2012. A follow-up review looking specifically at inflammation and hepatocellular necrosis was also performed in November 2012.

OBJECTIVE OF SLIDE REVIEW: The objective of the slide review of the National Toxicology Program's (NTP) study of 1,4-dioxane in mice was to determine if any non-neoplastic lesions in the liver were present in an effort to understand the sequence of events that may have contributed to the mode of action of the observed liver tumors in mice.

Another reason for the slide review was because at the time of the original slide review (1973) the NTP typically recorded only the most severe diagnosis on a given slide, e.g. adenoma or carcinoma. The focus of the study at this period of time was to determine potential carcinogenic activity of the chemical, not its potential chronic toxicity. For example, if an adenoma, carcinoma and evidence of chronic toxicity (e.g. hepatocellular hypertrophy), were all present on a given slide, only the tumor response was typically recorded. Thus, it was unclear whether non-neoplastic lesions were present in the livers of mice but not recorded in the NTP carcinogenicity study. Without this critical data it is next to impossible to determine the temporal sequence of events in the observed carcinogenicity of 1,4-1,4-dioxane using the NTP pathology tables.

APPROACH TO SLIDE REVIEW: An initial review of the livers from five control male mice was conducted followed by five male mice from the high dose. The reason for looking at the control livers first was to get an appreciation for what was normal in the liver of a mouse of this strain and age. Similarly, the five high dose mice were examined to understand the spectrum of lesions that occurred as a result of exposure to 1,4-dioxane after 2-years of exposure. The remainder of all of the other

[1] Bioassay Of 1,4-Dioxane For Possible Carcinogenicity CAS No. 123-91-1. National Cancer Institute, Carcinogenesis Technical Report Series, NO. 80, 1978.

male mice and female mice from the carcinogenicity study were examined in a "blinded" fashion, i.e. no knowledge of dose.
It should be noted that in some cases the liver could not be evaluated for non-neoplastic lesions because not enough tissue was available due to tumor involvement or postmortem autolysis. In such cases these animals were deleted from the "N" for statistical evaluations.

RESULTS: My evaluation of the various lesions found in this re-evaluation[2] are summarized below and in the tables following the report.

Depletion of hepatocellular glycogen – There appeared to be a distinct depletion of glycogen in the 1,4–dioxane exposed rats. However, it was difficult to discern a dose-response (i.e., a difference between the low and high-dose). In addition, depletion was also noted in many control rats, probably due to various causes, e.g. inanition and chronic disease of various types.

Hepatocellular hypertrophy – There was a very clear dose-response for this endpoint, especially in female mice. In affected livers most of the hepatocytes were diffusely enlarged. In cases with minimal hypertrophy, the affected hepatocytes were more apparent in the central lobular areas near the central vein.

Necrosis (particularly hepatocellular) – Dose-related hepatocellular necrosis was apparent in most of the exposed animals, manifested as isolated diffusely scattered necrotic hepatocytes. Most of the necrotic hepatocytes were centrilobular, particularly near the central veins.

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[2] Lesions found in the reread:
Depletion of hepatocellular glycogen – The hepatocytes in a normal liver contain readily recognizable amounts of glycogen which in an H&E slide appear as "empty" spaces in the cytoplasm of a normal sized cell. If the glycogen is depleted the hepatocytes do not contain empty spaces with the cytoplasm being more homogeneous.
Hepatocellular hypertrophy – Hypertrophic hepatocytes are conspicuously larger than normal due to an increase in the amount of cytoplasm. It is also typically more eosinophilic and devoid of recognizable glycogen. When hypertrophy is present an attempt will be made to determine if there was a zonal predilection, e.g. periportal, midzonal, centrilobular or diffuse.
Necrosis (particularly hepatocellular) – Hepatocellular necrosis is evidenced by increased cytoplasmic eosinophilia and disintegration of cytoplasm and cell membranes. Nucleii are often still apparent.
Inflammation – Inflammation is primarily evidenced by the focal influx of neutrophils and lymphocytes, primarily in the area of hepatocellular necrosis.
Fatty infiltration – Fatty infiltration is evidenced as individual hepatocytes containing round clear vacuoles.
Non-neoplastic hyperplasia (e.g. focal hyperplasia of several types: Kupffer cell, bile duct, and basophilic, eosinophilic, clear cell and mixed cell hyperplastic foci). Kupffer cell hyperplasia is typically recognized as diffuse proliferation of Kupffer cells. Bile duct hyperplasia is similarly recognized as multifocal proliferation of bile ducts. In contrast, basophilic, eosinophilic, clear-cell and mixed-cell foci are recognized as focal clonal-like accumulations of normal appearing hepatocytes with tinctorial qualities that allow for the specific morphological classifications. Importantly, the various types of hepatocellular foci are considered preneoplastic changes.
Various types of neoplasms - hepatocellular adenoma and carcinoma, leukemia, lymphoma, etc. will be diagnosed using standard morphological criteria.

Inflammation – Inflammation was micro-focal and was primarily in reaction to the necrosis of individual hepatocytes (described above). The appearance was somewhat unusual in that enlarged hepatocytes with almost normal appearing nucleii appeared to be invaded by neutrophils and lymphocytes. There was a definite dose-response.

Fatty infiltration – Fatty change was only rarely observed and there was no evidence of a treatment related effect.

Non-neoplastic hyperplasia (e.g. focal hyperplasia of several types):

> **Kupffer cell** – An increase in Kupffer cell hyperplasia was found in male mice and high dose female mice, and the response appeared to be dose related.
>
> **Bile Duct Hyperplasia** – Bile duct hyperplasia was only found in a few animals and only in exposed animals (data not shown, but available upon request). There were not enough affected mice to make any definite conclusions regarding dose-effects for this endpoint.
>
> **Basophilic, eosinophilic, clear cell and mixed cell foci** – These specific types of foci were observed in a dose-related pattern, especially when the various types of foci are combined. Interestingly, the hepatocytes in these clonal expansions are generally of normal size, i.e. not enlarged (hypertrophic), as are the hepatocytes surrounding the foci.

Various types of neoplasms, e.g. hepatocellular adenoma and carcinoma, leukemia, lymphoma, etc. – There was nothing unusual about these neoplasms other than an increased incidence in the treated rats. Also, the tumor counts closely match those of the NTP.

Male versus female – In general, the non-neoplastic lesions in the male mice were more apparent than in the females, but this may be due to the fact that the low dose female mice had only about ½ of the dose of the low dose male mice. The high doses in both sexes gave roughly comparable results, except for Kupffer cell hyperplasia.

DISCUSSION: Groups of 50 B6C3F1 mice, of each sex, were administered 1,4-dioxane at concentrations of either 0.5% or 1.0% (v/v) in the drinking water for 90 weeks with matched controls. Based on the measurements of water consumption and bodyweights, average daily intakes of 1,4-dioxane was 0, 720, and 830 mg/kg-day in male mice; and 0, 380, and 860 mg/kg-day in female mice. It is noteworthy that the dose of 1,4-dioxane consumed by the high and low doses males was similar and in the words of the authors, “did not reflect the twofold difference in concentration between the low and high doses”. Thus, the general similarity of the histologic pathology between the low and high males, wherein the pathology is only modestly more severe in the high dose males is likely attributable to the similar intake levels.

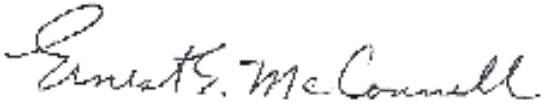
This reanalysis of the original NTP histology slides has provided histopathology that is on the whole similar to the liver histopathology observed following a 13-week drinking water exposure to Crj:BDF1

mice (Kano et al, 2008).[3] Similar to this reanalysis, 1,4-dioxane drinking water exposure to mice at doses of 86 to 1570 mg/kg-day for 13 weeks caused hepatocyte swelling and necrosis. This reanalysis is also similar to the results of a 2-year study in Crj:BDF1 mice (Kano et al, 2009) in that hepatocellular injury was evidenced in this investigation by the enhanced cytolytic release of liver enzymes (e.g. GOT, GPT, LDH, and ALP) at doses of 191 to 964 mg/kg-day.[4]

SUMMARY: In my opinion, this slide review supports the view that there are clearly identifiable dose-related non-neoplastic changes in the liver of mice exposed to 1,4–dioxane. The most clear examples of a dose-related effect are the hypertrophic response of hepatocytes, followed by necrosis/inflammation and hyperplastic hepatocellular foci.

If you have questions regarding this report, please feel free to contact me.

Sincerely,



Gene McConnell

[3] Kano, H; Umeda, Y; Saito, M; Senoh, H; Ohbayashi, H; Aiso, S; Yamazaki, K; Nagano, K; Fukushima, S. 2008. Journal of Toxicological Sciences Volume 33: 141-153.

[4] Kano, H; Umeda, Y; Kasai, T; Sasaki, T; Matsumoto, M; Yamazaki, K; Nagano, K; Arito, H; Fukushima, S. 2009. Food and Chemical Toxicology. Volume 47:2776-2784.

Table 1: Incidences of Selected Nonneoplastic Lesions in Mice (with recounted data for necrosis and inflammation)

	0-No lesion (%)	1-Minimal (%)	2-Mild (%)	3-Moderate (%)	4-Marked (%)	Total (%)
MALE						
Hypertrophy						
Control (0 mg/kg-day)	41 (93)	2 (4.5)	1 (2.3)	0	0	**3/44 (6.8)**
Low (720 mg/kg-day)	2 (4.7)	17 (40)	24 (56)	0	0	**41/43 (95)**
High (830 mg/kg-day)	1 (2.4)	13 (31)	27 (64)	1 (2.4)	0	**41/42 (98)**
Necrosis						
Control (0 mg/kg-day)	44 (92)	4 (8.3)	0	0	0	**4/48 (8.3)**
Low (720 mg/kg-day)	4 (9.8)	16 (39)	16 (39)	5 (12)	0	**37/41 (90)**
High (830 mg/kg-day)	7 (18)	20 (50)	10 (25)	3 (7.5)	0	**33/40 (83)**
Inflammation						
Control (0 mg/kg-day)	44 (92)	4 (8.3)	0	0	0	**4/48 (8.3)**
Low (720 mg/kg-day)	4 (9.8)	17 (41)	16 (39)	4 (9.8)	0	**37/41 (90)**
High (830 mg/kg-day)	8 (20)	19 (48)	10 (25)	3 (7.5)	0	**32/40 (80)**
Kupffer cell hyper						
Control (0 mg/kg-day)	41 (93)	2 (4.5)	1 (2.3)	0	0	**3/44 (6.8)**
Low (720 mg/kg-day)	14 (33)	20 (47)	8 (19)	1 (2.3)	0	**29/43 (67)**
High (830 mg/kg-day)	11 (26)	15 (36)	13 (31)	3 (7.1)	0	**31/42 (74)**

	0-No lesion (%)	1-Minimal (%)	2-Mild (%)	3-Moderate (%)	4-Marked (%)	Total (%)
			FEMALE			
Hypertrophy						
Control (0 mg/kg-day)	46 (100)	0	0	0	0	**0/46 (0)**
Low (380 mg/kg-day)	20 (54)	14 (38)	3 (8.1)	0	0	**17/37 (46)**
High (860 mg/kg-day)	1 (3.3)	14 (47)	12 (40)	3 (10)	0	**29/30 (97)**
Necrosis						
Control (0 mg/kg-day)	19 (41)	25 (54)	2 (4.3)	0	0	**27/46 (59)**
Low (380 mg/kg-day)	20 (54)	14 (38)	2 (5.4)	1 (2.7)	0	**17/37 (46)**
High (860 mg/kg-day)	2 (11)	12 (63)	5 (26)	0	0	**17/19 (90)**
Inflammation						
Control (0 mg/kg-day)	20 (44)	24 (52)	2 (4.3)	0	0	**26/46 (57)**
Low (380 mg/kg-day)	20 (54)	14 (38)	2 (5.4)	1 (2.7)	0	**17/37 (46)**
High (860 mg/kg-day)	3 (16)	11 (58)	5 (26)	0	0	**16/19 (84)**
Kupffer cell hyper						
Control (0 mg/kg-day)	46 (100)	0	0	0	0	**0/46 (0)**
Low (380 mg/kg-day)	36 (97)	1 (2.7)	0	0	0	**1/37 (2.7)**
High (860 mg/kg-day)	21 (70)	5 (17)	2 (6.7)	1 (3.3)	1 (3.3)	**9/30 (30)**

Table 2: Average Severity Score

	Hypertrophy	Necrosis	Inflammation	KCH
MALE				
Control	0.1	0.08	0.08	0.1
Low	1.5	1.5	1.5	0.9
High	1.7	1.2	1.2	1.2
FEMALE				
Control	0	0.6	0.6	0
Low	0.5	0.6	0.6	0.03
High	1.6	1.2	1.1	0.5

Table 3: Glycogen Incidences

	n	2 = Normal (%)	1 = Decreased /Minimal (%)	0 = No Glycogen (%)	Average Score
MALE					
Control	44	14 (32)	19 (43)	11 (25)	1.1
Low	43	5 (12)	6 (14)	32 (74)	0.4
High	42	1 (2.4)	6 (14)	35 (83)	0.2
FEMALE					
Control	46	8 (17)	20 (44)	18 (39)	0.8
Low	37	7 (19)	13 (35)	17 (46)	0.7
High	30	3 (10)	6 (20)	21 (70)	0.4

Table 4: Foci Incidences (B = basophilic; E = eosinophilic; CC = clear cell; MC = mixed cell)

	n	B Focus (%)	E Focus (%)	CC Focus (%)	MC Focus (%)	Total Foci (%)
MALE						
Control	44	2 (4.5)	0	2 (4.5)	0	4/44 (9.1)
Low	43	6 (14)	2 (4.7)	2 (4.7)	3 (7.0)	13/43 (30)
High	42	2 (4.8)	0	4 (9.5)	1 (2.4)	7/42 (17)
FEMALE						
Control	46	0	1 (2.2)	0	0	1/46 (2.2)
Low	37	1 (2.7)	5 (14)	2 (5.4)	2 (5.4)	10/37 (27)
High	30	1 (3.3)	2 (6.7)	4 (13)	1 (3.3)	8/30 (27)

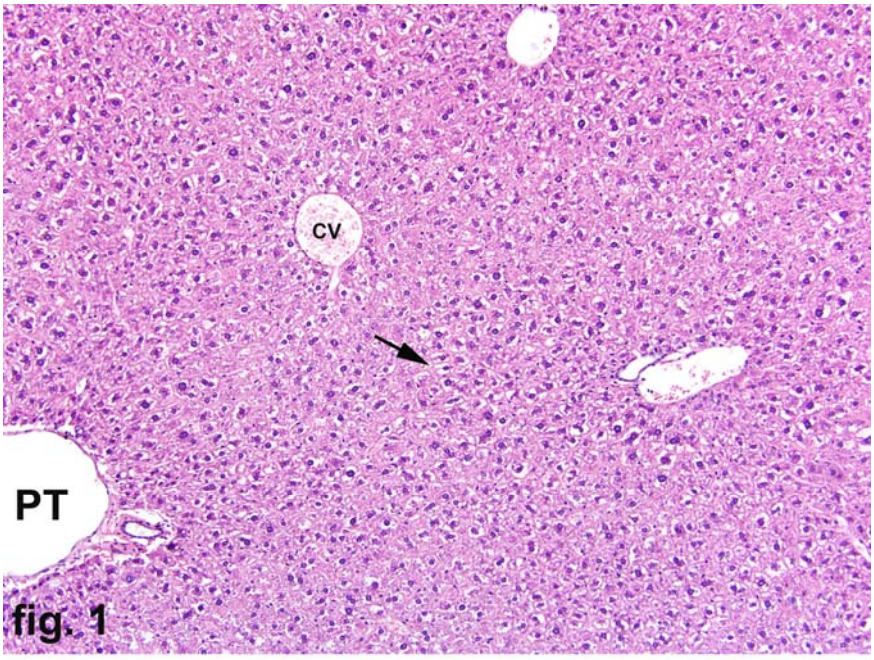
Table 5: Incidences of Animals with Neoplasms (%)[1]

	Adenoma		Carcinomas		Adenomas/ Carcinomas
	NTP	Recount	NTP	Recount	Recount
MALE					
Control	6/49	2/44 (4.5)	2/49	4/44 (9)	5/44 (11)
Low	1/50	1/48 (2)	18/50	16/48 (33)	17/48 (35)
High	4/47	3/48 (6)	24/47	21/48 (43)	22/48 (45)
FEMALE					
Control	0/50	0/49	0/50	0/49	0/49
Low	9/48	7/45 (16)	12/48	7/45 (16)	14/45 (31)
High	6/37	11/37 (30)	29/37	23/37 (62)	29/37 (78)

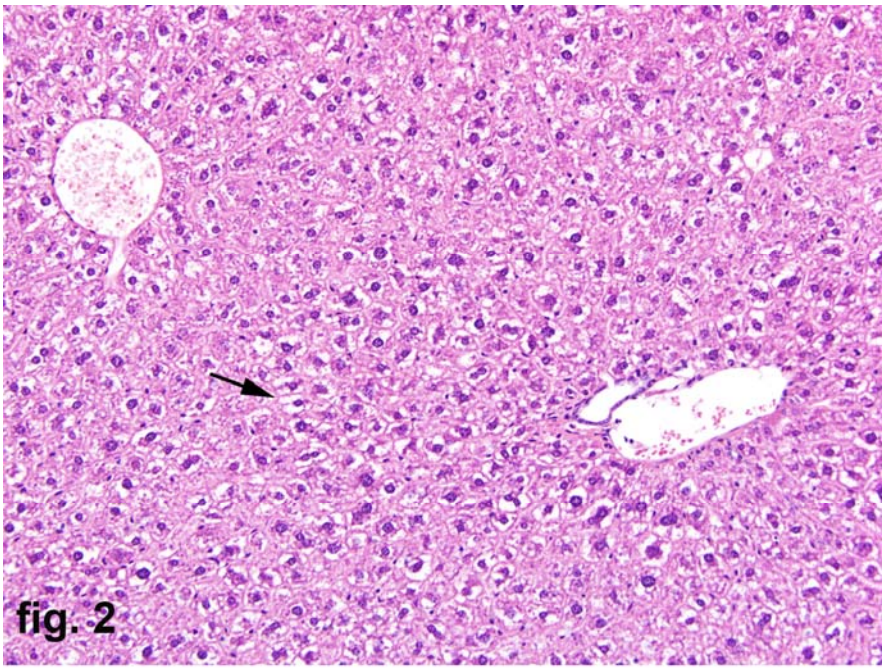
[1] In some cases the “n" between foci and tumor counts differ and in some cases the “n" between the tumor counts shown here and with NTP differ. In former case this is due to the fact that some tissues did not have enough non-tumor related tissue to make a judgment on foci. In the latter case this is due to the fact that the reread of a few slides was not possible, or in one case the reread allowed a larger “n.”

Appendix A – Photomicrographs

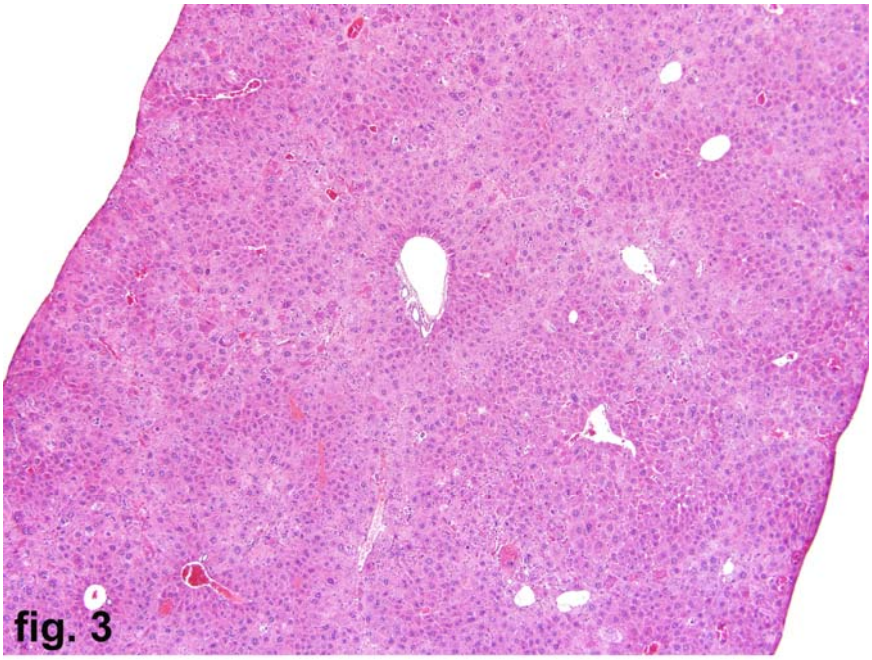
FIGURE #	PHOTO. #	CHEMICAL NAME	CHEM. #	TDMS #	SPECIES	SPECIES STRAIN	GROUP	ANI. #	HISTO. #	TISSUE	LESION	MAG.
FIG. 1	A72476	1,4-DIOXANE	C03689B	N/A	M3	B6C3F1	UM (GROUP #11712)	4	74-89	LIVER	NORMAL GLYCOGEN	10
FIG. 2	A72477	1,4-DIOXANE	C03689B	N/A	M3	B6C3F1	UM (GROUP #11712)	4	74-89	LIVER	NORMAL GLYCOGEN	16
FIG. 3	A72478	1,4-DIOXANE	C03689B	N/A	M3	B6C3F1	1%M (GROUP #12912)	1	73-2723	LIVER	HYPERTROPHY	4
FIG. 4	A72479	1,4-DIOXANE	C03689B	N/A	M3	B6C3F1	1%M (GROUP #12912)	1	73-2723	LIVER	HYPERTROPHY AND NECROSIS	10
FIG. 5	A72480	1,4-DIOXANE	C03689B	N/A	M3	B6C3F1	1%M (GROUP #12912)	1	73-2723	LIVER	NECROSIS AND INFLAMMATION	32
FIG. 6	A72481	1,4-DIOXANE	C03689B	N/A	M3	B6C3F1	1%M (GROUP #12912)	1	73-2723	LIVER	NECROSIS AND INFLAMMATION	20
FIG. 7	A72482	1,4-DIOXANE	C03689B	N/A	M3	B6C3F1	1%M (GROUP #12912)	1	73-2723	LIVER	CLEAR CELL FOCUS	4
FIG. 8	A72483	1,4-DIOXANE	C03689B	N/A	M3	B6C3F1	1%M (GROUP #12912)	5	73-2726	LIVER	HEPATOCELLULAR ADENOMA	3.2
FIG. 9	A72484	1,4-DIOXANE	C03689B	N/A	M3	B6C3F1	1%M (GROUP #12912)	2	73-2724	LIVER	KUPFFER CELL HYPERPLASIA	10
FIG. 10	A72485	1,4-DIOXANE	C03689B	N/A	M3	B6C3F1	1%M (GROUP #12912)	5	73-2726	LIVER	HEPATOCELLULAR CARCINOMA	2
FIG. 11	A72486	1,4-DIOXANE	C03689B	N/A	M3	B6C3F1	0.5%M (GROUP #12922)	5	73-2772	LIVER	HEPATOCELLULAR CARCINOMA	2
FIG. 12	A72487	1,4-DIOXANE	C03689B	N/A	M3	B6C3F1	0.5%M (GROUP #12922)	5	73-2772	LIVER	HEPATOCELLULAR CARCINOMA	4
FIG. 13	A72488	1,4-DIOXANE	C03689B	N/A	M3	B6C3F1	0.5%M (GROUP #12922)	32	73-2798	LIVER	EOSINOPHILIC FOCUS	4
FIG. 14	A72489	1,4-DIOXANE	C03689B	N/A	M3	B6C3F1	0.5%M (GROUP #12922)	48	73-2813	LIVER	MIXED CELL FOCUS	4
FIG. 15	A72490	1,4-DIOXANE	C03689B	N/A	M3	B6C3F1	UM (GROUP #11712)	40	74-123	LIVER	BASOPHILIC FOCUS	2
FIG. 16	A72491	1,4-DIOXANE	C03689B	N/A	M3	B6C3F1	UM (GROUP #11712)	40	74-123	LIVER	BASOPHILIC FOCUS	4



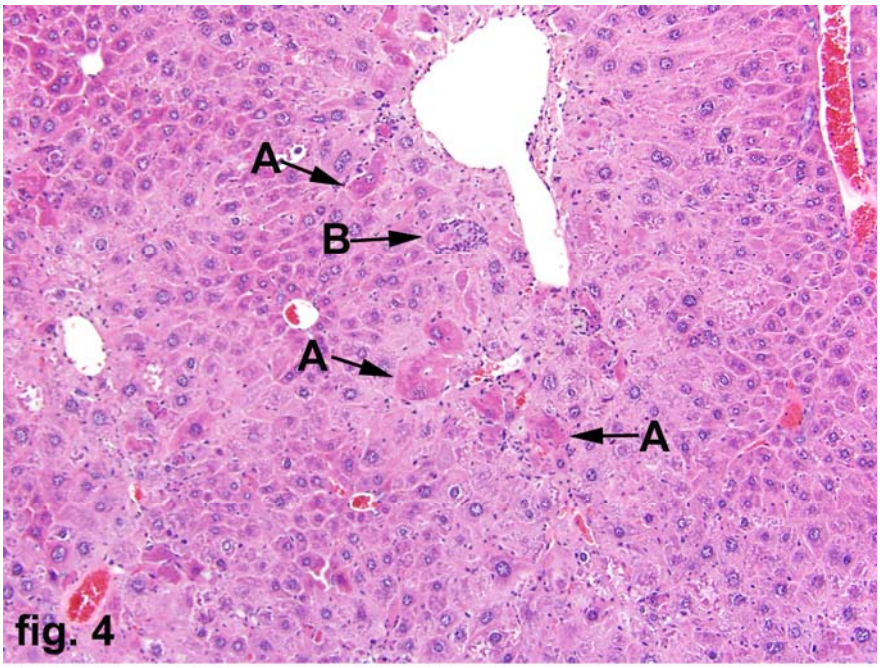
Liver, control - hepatocytes showing normal amounts of glycogen (arrow).
PT=Portal tract. CV=Central vein. 10x



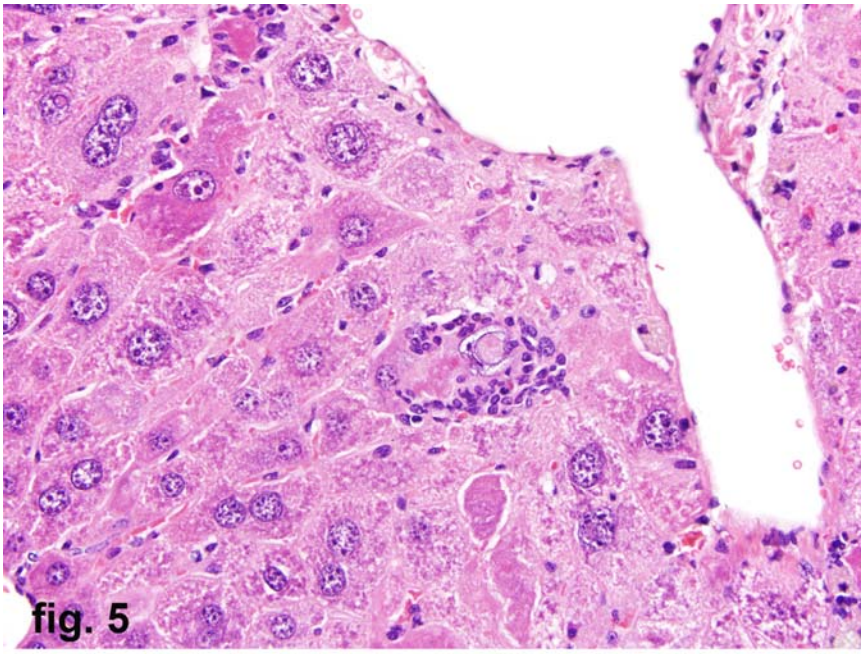
Higher magnification of figure 1 of glycogen containing hepatocytes. 16x



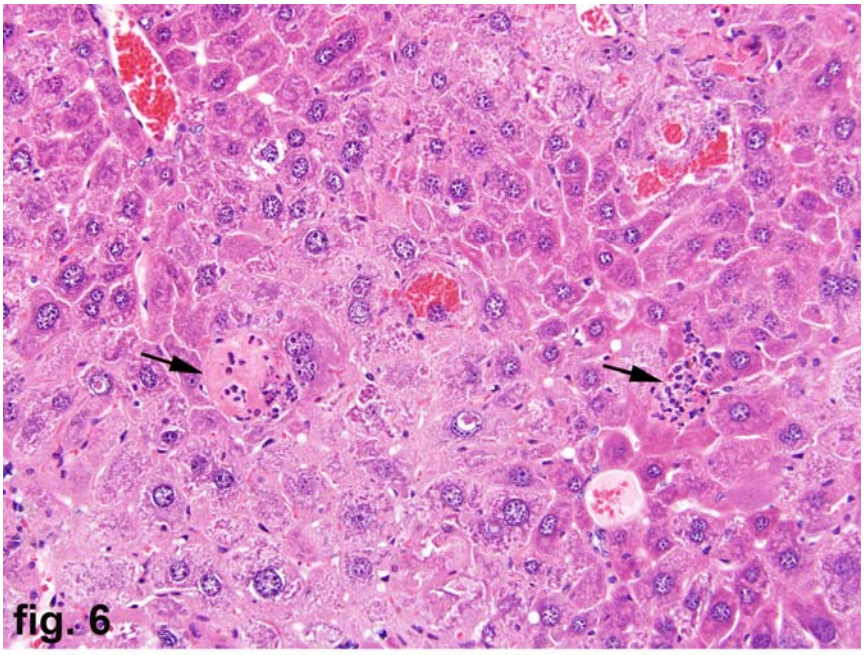
1% 1,4-dioxane - Grade 2 diffuse hepatocellular hypertrophy. 4x



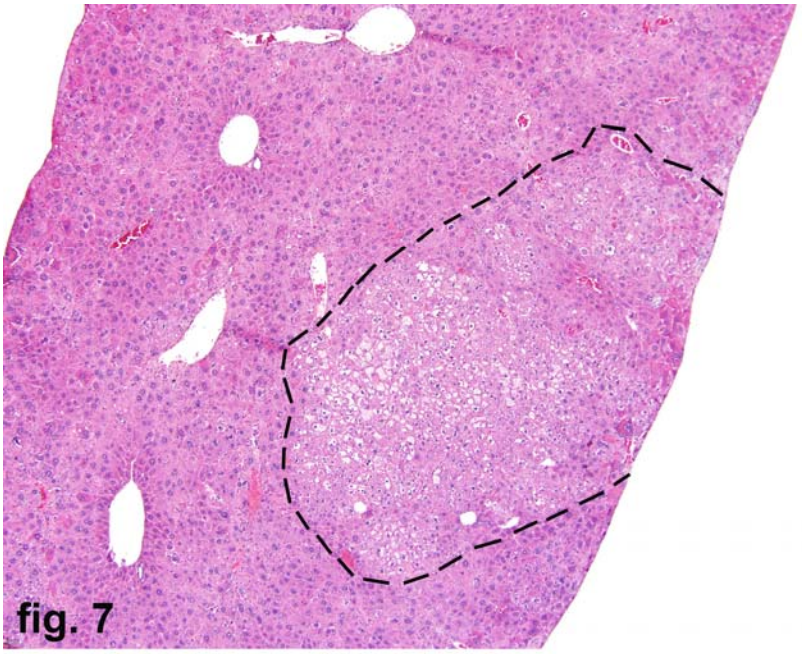
1% 1,4-dioxane - Higher magnification of figure 3 showing hepatocellular necrosis (A) and inflammation (B). 10x



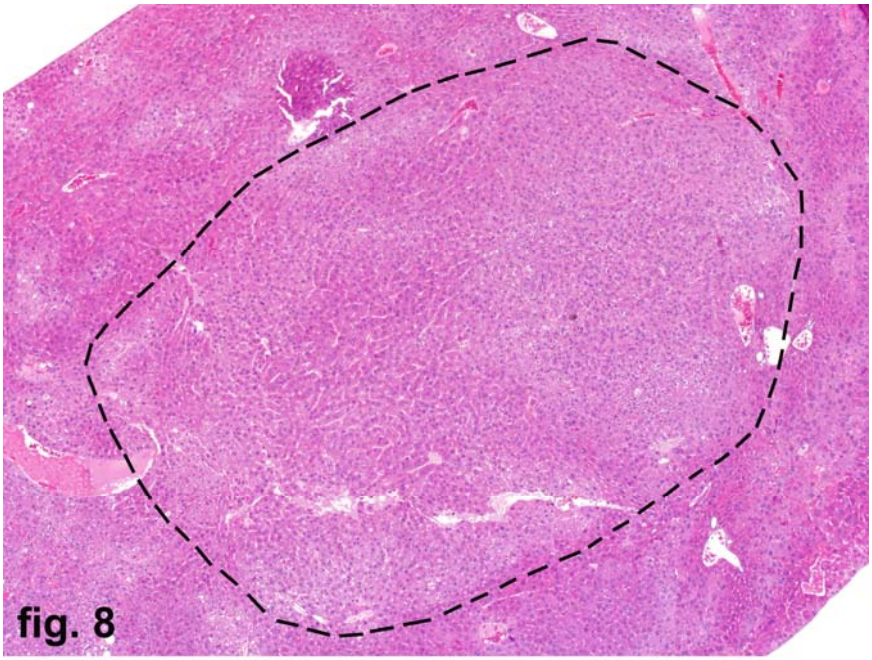
Higher magnification of figure 4. 32x



Same animal as figure 4 with foci of single cell inflammation (arrows). 20x



1% 1,4-dioxane - Clear cell focus. 4x



1% 1,4-dioxane - Hepatocellular adenoma. 3.2x

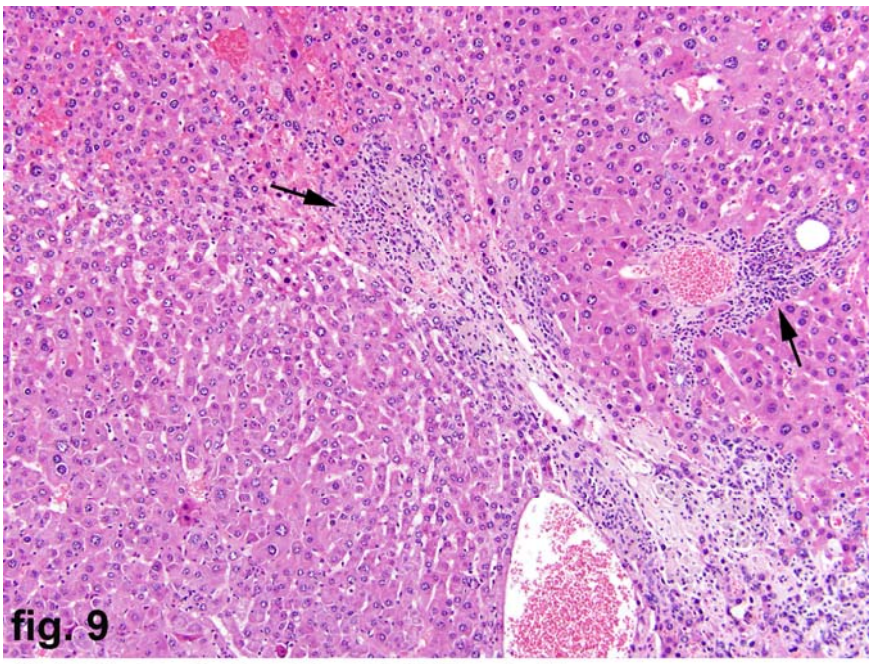


fig. 9

1% 1,4-dioxane - Kupffer cell hyperplasia (arrows). 10x

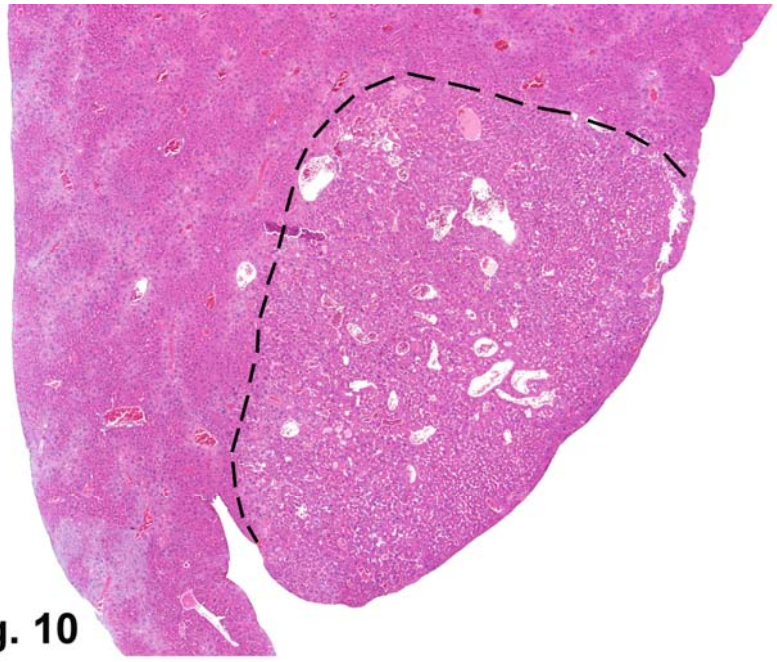
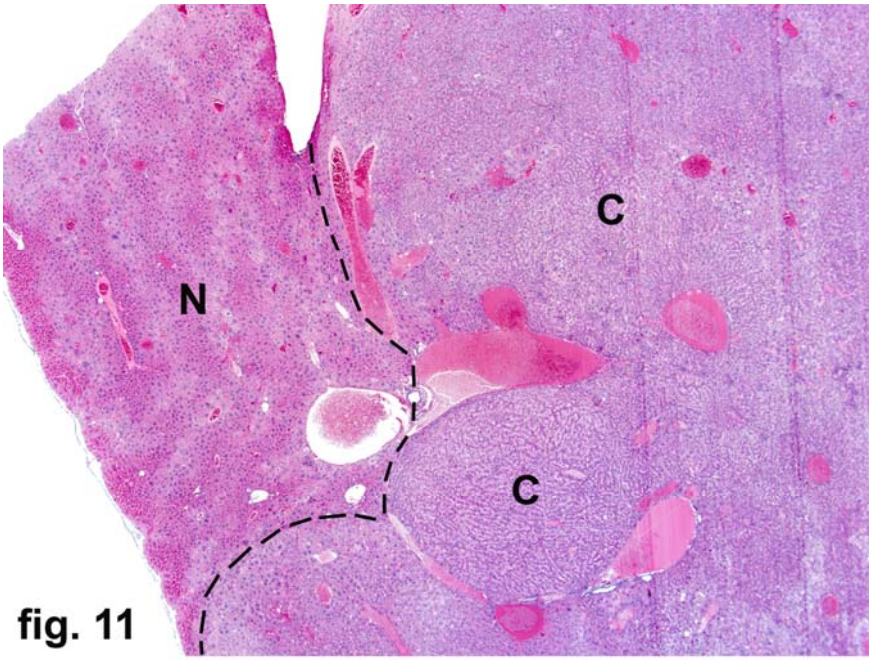
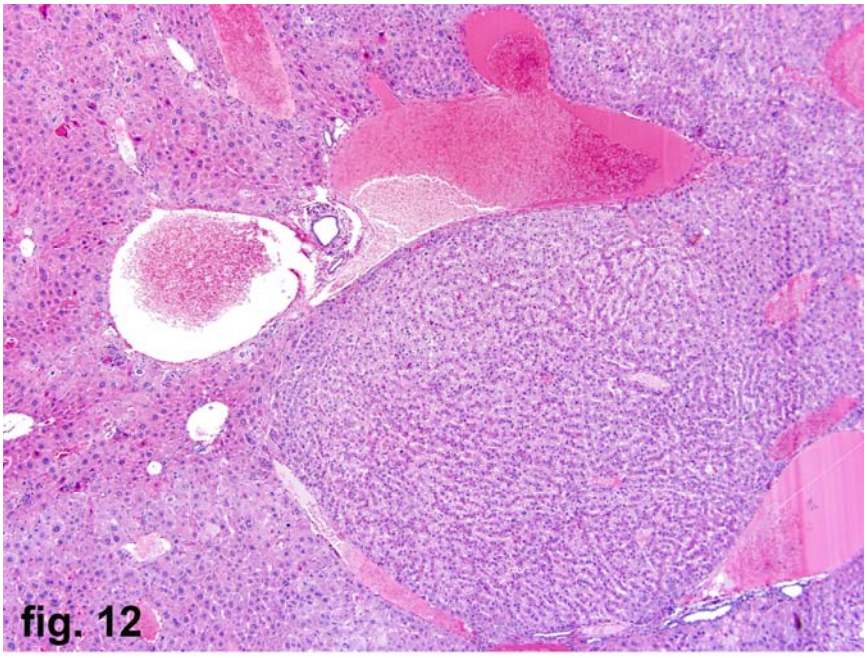


fig. 10

1% 1,4-dioxane - Small hepatocellular carcinoma. 2x



0.5% 1,4-dioxane - Large hepatocellular carcinoma (C). N = Non-tumor liver. 2x



Higher magnification of figure 11 showing trabecular pattern. 4x

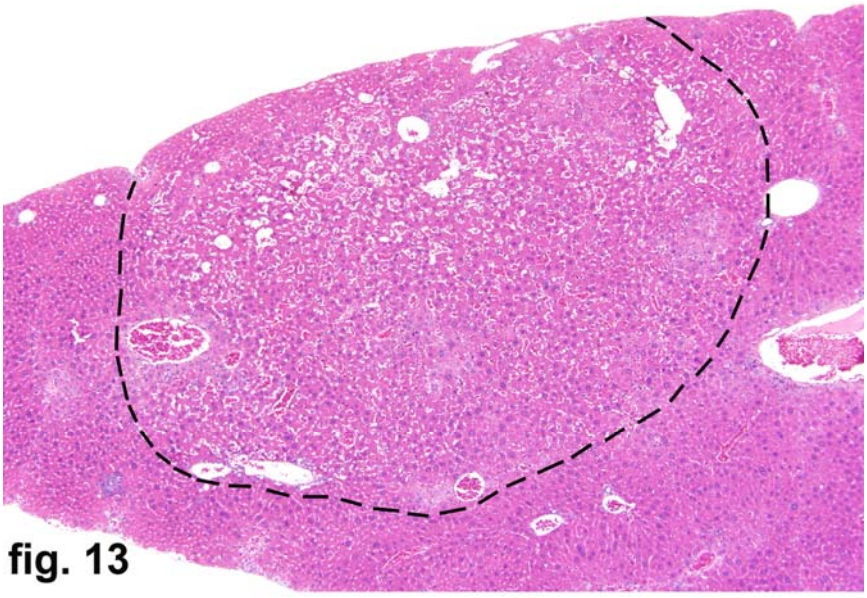


fig. 13

0.5% 1,4-dioxane - Eosinophilic focus. 4x

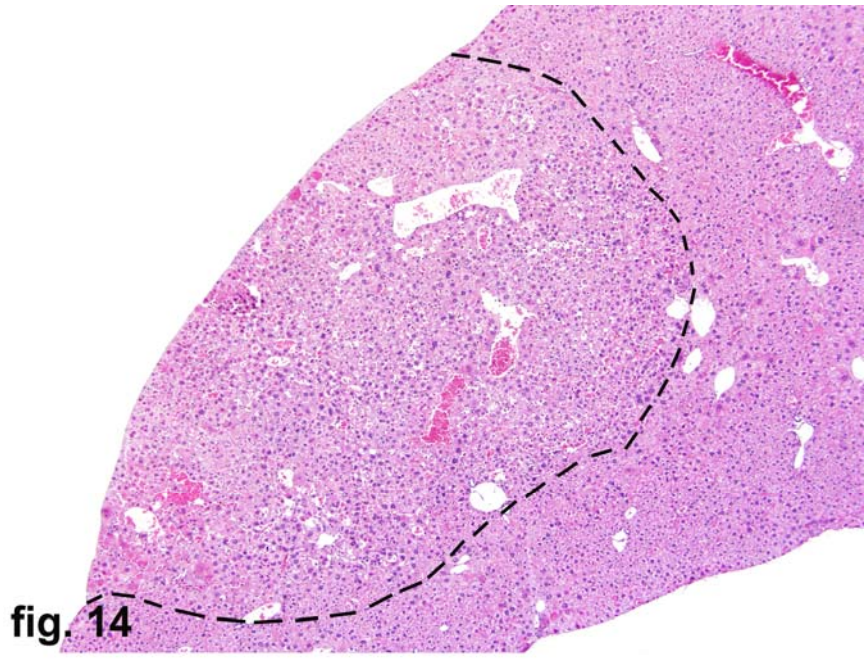


fig. 14

0.5% 1,4-dioxane - Mixed cell focus. 4x

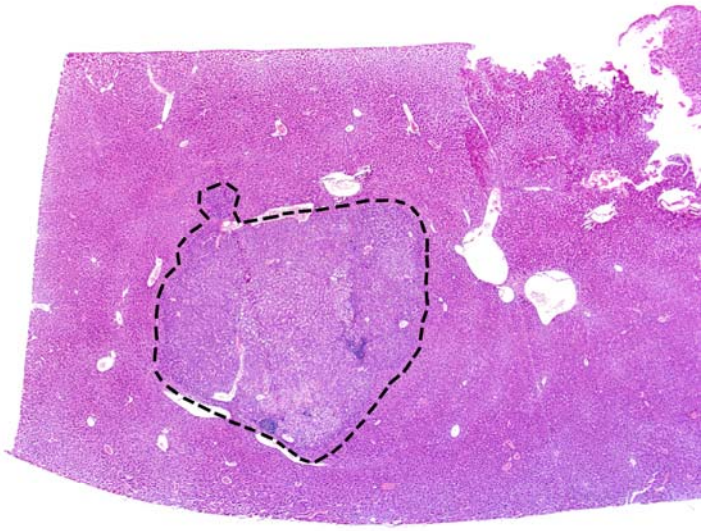


fig. 15

Control 1,4-dioxane - Basophilic focus 2x

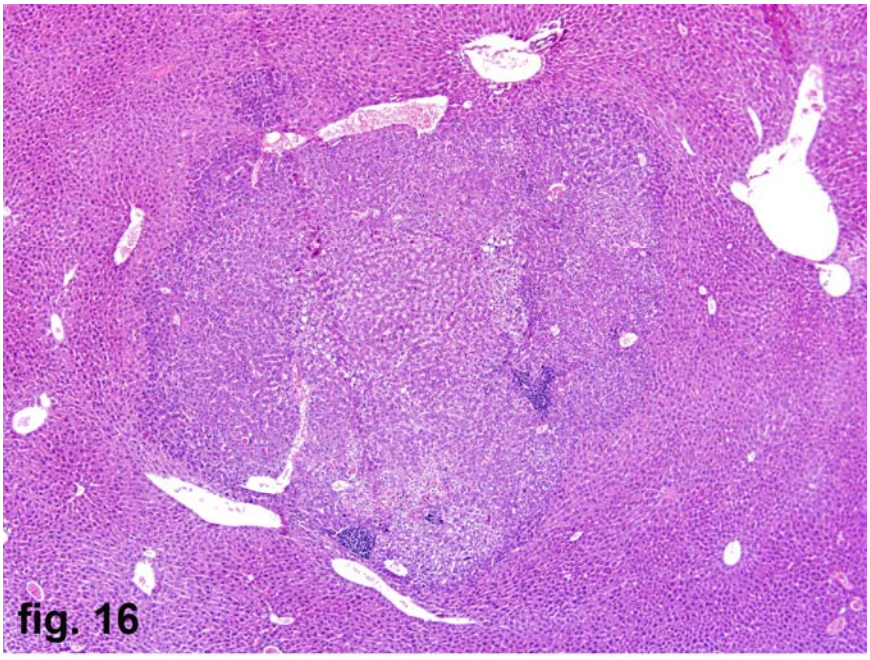


fig. 16

Higher magnification of figure 15. 4x