

## QUANTITATIVE STUDY OF COLLAGEN CONTENT IN EXPERIMENTAL CIRRHOSIS\*

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PLATES 8 AND 9

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It has been noted that experimental cirrhosis undergoes a reversal when the administration of hepatotoxic agents is discontinued. This recovery or restitution phenomenon has been described in cirrhosis due to *p*-dimethylaminoazobenzene by Orr (1), and in carbon tetrachloride cirrhosis by Cameron and Karunaratne (2). The latter have also shown that the prolonged administration of carbon tetrachloride produces irreversible cirrhosis.

In 1941 a chemical quantitative method of determining collagen in tissues was introduced by Lowry, Gilligan, and Katersky (3). This method has been applied to experimental cirrhosis in this present study as a means of evaluating the extent of deposition and disappearance of collagen. Parallel histological observations on the same material were made in order to determine whether the morphological and chemical data could be correlated.

### *Methods*

Hepatic cirrhosis was produced in rats with carbon tetrachloride and *p*-dimethylaminoazobenzene. A total of one hundred and nine rats was utilized. Male albino rats weighing 120 to 140 gm. each were employed in both groups. Carbon tetrachloride was administered by inhalation of vapors by a modification of the method described by Forbes and Neal (4) and Morrione (5). Fifty-six rats were so treated for 20 minutes three to four times a week for 3½ months. At this time twenty-five animals were autopsied. Administration of carbon tetrachloride was stopped on the remaining thirty-one rats. These animals were then killed as follows: ten after 1 month, twelve after 2 months, and nine after 3 months.

The butter yellow (*p*-dimethylaminoazobenzene) was fed to a group of thirty-eight rats. The drug, in 3 per cent olive oil solution, was mixed with coarsely ground brown rice<sup>1</sup> in a concentration of 0.06 per cent (6). This basal diet was supplemented with approximately 1 gm. per rat of fresh carrot daily. After 5½ months twenty-three rats were killed, and the remaining animals were fed on a normal diet (dog chow pellets) and killed after 1½ months.

Quantitative collagen determinations were performed according to Lowry's method on representative portions of livers of all autopsied animals, including fifteen normal adult male rats. The samples of liver chosen for analysis weighed approximately 3 gm. The chemical

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<sup>1</sup> Comet brown rice supplied by Comet Rice Mills, Beaumont, Texas.

procedure in brief consists of extracting thoroughly ground liver with  $N/10$  NaOH and fat solvents. This extraction removes substances other than collagen and elastin. The collagen is separated and removed from the elastin in the residue by autoclaving. The latter procedure selectively and quantitatively converts the collagen in the residue into gelatin. The weight of collagen in the sample analyzed is represented by the difference in the dry weight of the residue before and after autoclaving. All animals and livers were weighed. Representative sections of the livers were stained with hematoxylin-eosin, hematoxylin-eosin-azure, Masson's trichrome, and Gomori's reticulum stain (7).

#### RESULTS

The average hepatic collagen content in a group of fifteen normal rats was 0.23 per cent wet weight, with values ranging from 0.18 per cent to 0.29 per cent. The total hepatic collagen was determined by multiplying the weight of each liver by the corresponding per cent collagen value. The total hepatic collagen in this normal group varied from 18.4 to 30.5 mg., with an average value of 23.2 mg.

Animals receiving *p*-dimethylaminoazobenzene showed a well developed cirrhosis after 5 months on the experimental diet (Figs. 2 and 4). The increases in collagen occurring in these livers are shown in Table I. The collagen content rose to approximately twice its normal value. The increase in average total hepatic collagen to 47.5 mg. per liver shows this twofold increase to represent an absolute rather than a relative change. Histologically the collagen was present for the most part in periportal regions, in newly formed fibrous septae, and as a more delicate network surrounding groups of hepatic cells. The alterations occurring in cirrhosis due to *p*-dimethylaminoazobenzene have been previously described (1, 8, 9). In our present series hepatic cell carcinoma was seen in three animals, bile duct carcinoma in one, hepatomata in twelve, and bile duct adenomata and cystadenomata in twelve. Only one animal (Table I) showed a grossly bulky tumor, and in this instance the specimen for collagen determination was selected from hepatic regions which showed no tumor tissue visible in the gross.

The decrease in hepatic collagen content occurring in the group fed a normal diet (dog chow pellets) for  $1\frac{1}{2}$  months following cessation of the *p*-dimethylaminoazobenzene-rice diet is shown in Table I. That this fall represents an actual removal of collagen from the livers is shown by the decrease in total hepatic collagen from 47.5 mg. per liver in the group with cirrhosis, to 28.3 mg. in the "reversal" group. Microscopic study of the livers of the latter group stained with Masson trichrome showed scattered small amounts of residual collagen in ten out of the fifteen animals. However, reticulum stain of the same livers revealed moderate amounts of residual argyrophilic reticulum (Fig. 6). Other residual alterations occurring in this group were hepatomata in three animals and bile duct adenomata in two animals.

TABLE I  
*Cirrhosis Due to p-Dimethylaminoazobenzene*

Rat No.	Rat weight	Liver weight	Collagen wet weight	Total hepatic collagen	Microscopic evaluation of collagen, based upon reticulum-stained sections
	<i>gm.</i>	<i>gm.</i>	<i>per cent</i>		
1			0.73		
2			0.33		
3	87.	3.1	0.49	15.1	++
4	112.7	5.3	0.61	32.0	++++
5	118.4	4.9	0.71	35.1	++++
6	127.1	7.3	0.35	25.5	+
7	139.5	7.9	0.50	39.9	++++
8	149.6	8.9	0.51	45.1	+++
9	151.6	9.5	0.46	43.5	++
10	159.4	13.0	0.56	72.9	+++
11	161.0	8.3	0.28	23.4	++
12	183.4	7.5	0.43	32.2	+
13	184.4	10.0	0.49	49.1	+++
14	191.8	33.6*	0.48	161.3	+++
15	193.3	9.4	0.43	40.3	++
16	198.1	11.3	0.31	35.0	+++
17	204.5	9.4	0.47	44.0	+++
18	217.4	9.7	0.34	33.0	++
19	218.0	10.1	0.85	85.5	++++
20	219.0	10.6	0.44	46.5	++
21	238.7	11.1	0.42	46.6	+++
22	244.0	7.9	0.34	27.0	++
23	284.4	13.2	0.49	64.4	+++
Average . . .	180.1	10.1	0.48†	47.5	

Reversal of cirrhosis group. On normal§ diet for 1½ months following cessation of p-dimethylaminoazobenzene-rice diet.

26	213.0	10.6	0.24	25.4	+
25	215.8	9.0	0.13	11.7	0
26	216.5	8.9	0.28	24.9	+
27	218.6	10.6	0.14	14.8	0
28	229.0	10.0	0.24	24.0	+

\* Large bulky primary hepatic carcinoma.

† Standard deviation equals 0.13 (animals 1 to 23).

§ Dog chow pellets.

TABLE I—*Concluded*

Rat No.	Rat weight	Liver weight	Collagen wet weight	Total hepatic collagen	Microscopic evaluation of collagen, based upon reticulum-stained sections
Reversal of cirrhosis group. On normal§ diet for 1½ months following cessation of <i>p</i> -dimethylaminoazobenzene-rice diet.— <i>Continued.</i>					
	<i>gm.</i>	<i>gm.</i>	<i>per cent</i>		
29	246.2	10.6	0.19	20.1	+
30	257.0	9.2	0.28	25.8	+
31	262.6	12.8	0.23	29.4	+
32	291.1	11.6	0.22	25.5	0
33	295.2	12.6	0.30	37.8	0
34	298.4	12.2	0.34	41.5	+
35	307.3	12.0	0.24	28.8	+
36	314.5	15.4	0.31	47.7	+
37	329.0	12.1	0.29	35.1	+
38	335.9	15.5	0.21	32.6	0
Average . . .	268.7	11.5	0.24	28.3	

*Cirrhosis Due to Carbon Tetrachloride*

The increases in collagen occurring in this group (Table II) were greater than those in cirrhosis due to *p*-dimethylaminoazobenzene. The average per cent collagen content rose to 1.01 per cent, and the total hepatic collagen averaged 86.4 mg. per liver. These high levels of collagen were due to advanced hepatic cirrhosis, as determined microscopically (Figs. 1 and 3). In many livers the parenchyma was reduced to mere small scattered islands separated by broad sheets of fibrous tissue. The latter contained many histiocytes, fibroblasts, lymphocytes, and proliferating bile ducts. This severe degree of cirrhosis resulted from the prolonged, heavy exposure to the carbon tetrachloride vapors. Cameron and Karunaratne (2) have shown that the prolonged administration of carbon tetrachloride results in only partially reversible, or irreversible cirrhosis. The decrease in collagen content following cessation of carbon tetrachloride in our group was accordingly slow, gradual, and incomplete (Fig. 5).

The greatest decrease occurred in the 1st month (Table II) during which time the average per cent collagen content fell from 1.01 per cent to 0.69 per cent, and the average total hepatic collagen dropped from 86.4 to 73.7 mg. After two additional months of reversal the levels had fallen to 0.51 per cent

TABLE II  
*Cirrhosis Due to Carbon Tetrachloride*

Rat No.	Rat weight	Liver weight	Collagen wet weight	Total hepatic collagen	Microscopic evaluation of collagen, based upon reticulum-stained sections
	<i>gm.</i>	<i>gm.</i>	<i>per cent</i>		
1			1.14		
2			1.27		
3			1.36		
4			0.89		
5	112.0	2.6	1.10	28.4	++++
6	139.8	5.2	1.32	69.2	++++
7	154.6	5.0	1.09	54.5	++++
8	158.1	9.1	1.83	166.5	++++
9	161.2	7.9	0.73	57.7	++
10	161.7	8.5	0.97	82.5	++++
11	163.5	8.4	1.82	152.9	++++
12	167.5	7.8	0.64	49.9	++++
13	172.6	8.9	0.77	69.1	++++
14	176.6	8.9	0.99	88.1	+++
15	182.7	10.2	0.73	74.5	+++
16	183.3	8.8	0.99	87.1	+-
17	187.4	11.5	0.59	67.9	+++
18	197.5	9.6	1.48	111.1	++++
19	200.1	9.8	1.23	120.5	+
20	200.9	9.1	1.44	131.0	+++
21	208.2	7.8	0.57	44.5	+-
22	209.8	10.0	0.79	78.9	+++
25	210.0	10.1	1.15	116.2	++++
24	227.4	9.6	0.89	85.4	+++
25	237.8	11.0	0.44	48.4	+
Average. . . .	181.6	8.6	1.01*	86.4	
<i>Reversal Group A</i>					
<i>Carbon Tetrachloride Discontinued for 1 Month</i>					
26	181.7	8.7	0.67	58.3	+++
27	199.7	8.6	0.69	59.3	++
28	206.5	7.8	0.64	49.9	+++
29	212.9	10.1	0.86	86.9	+++
30	216.0	8.8	0.61	53.7	+++
31	223.9	10.9	0.86	93.7	++++
32	246.5	10.6	0.57	64.2	++
33	280.5	10.9	0.49	53.4	+++
34	280.7	14.1	0.99	139.6	++++
35	296.2	13.5	0.58	78.3	+++
Average. . . .	234.5	10.4	0.69†	73.7	

\* Standard deviation, 0.14.

† Standard deviation, 0.17.

TABLE II—*Concluded**Reversal Group B**Carbon Tetrachloride Discontinued for 2 Months*

Rat No.	Rat weight	Liver weight	Collagen wet weight	Total hepatic collagen	Microscopic evaluation of collagen content, based upon reticulum-stained sections
	<i>gm.</i>	<i>gm.</i>	<i>per cent</i>		
36	153.5	6.5	0.61	39.7	++
37	155.7	7.9	0.84	66.4	++++
38	186.3	9.4	0.57	53.6	+++
39	201.3	8.6	0.68	58.5	+++
40	210.0	8.3	0.54	44.8	+++
41	227.0	10.0	0.68	68.0	+++
42	227.3	11.1	0.33	36.6	++
43	227.5	10.1	0.30	30.3	+
44	240.5	10.8	0.86	92.9	+++
45	263.2	11.0	0.56	61.6	+++
46	293.6	11.5	0.67	77.1	+++
47	299.8	13.5	0.64	86.4	+++
Average. . . .	223.8	9.9	0.61§	59.6	

*Reversal Group C**Carbon Tetrachloride Discontinued for 3 Months*

48	225.1	9.6	0.55	52.8	+++
49	231.4	12.6	0.42	52.9	++
50	244.4	11.4	0.39	44.5	+
51	257.9	13.4	0.50	67.0	++
52	273.8	11.5	0.49	56.4	++
53	285.7	13.3	0.49	65.2	++
54	301.6	14.3	0.59	84.4	+++
55	303.1	12.9	0.52	67.1	+++
56	309.5	12.8	0.60	76.8	+++
Average. . . .	270.7	12.4	0.51	63.0	

§ Standard deviation, 0.15.

|| Standard deviation, 0.01.

collagen and 63 mg. average total hepatic collagen (Chart 1). These groups were of interest because in them, the microscopic observation of persisting cirrhosis could be correlated with persisting high collagen values.

*Statistical Analysis of Chemical Data*

The standard deviation was determined for the various experimental groups. These are presented in Tables I and II. The significance of differences between the arithmetic means was evaluated by Fisher's "t" test. The latter

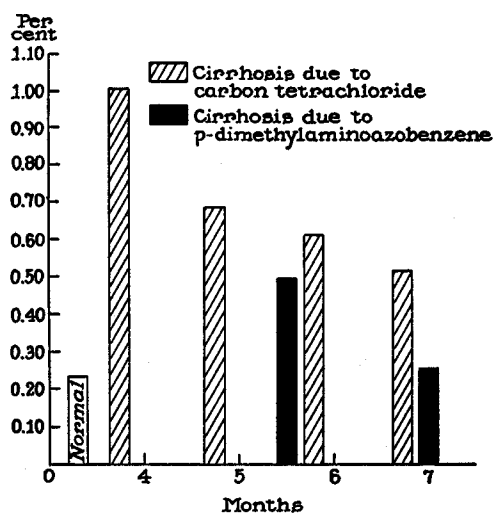


CHART 1. Graphic representation of alterations in collagen content in reversal of experimental cirrhosis.

showed the increases in collagen in the cirrhotic groups to be highly significant statistically. The decrease in per cent collagen content in the "reversal" groups is also highly significant. The average of 0.51 per cent (Table II) after 3 months, differs significantly from the 0.69 per cent value after 1 month's reversal, showing that the decrease in collagen which occurred during this period was progressive. It is worth mention, however, that the difference between 0.69 and 0.61 per cent is not statistically significant, nor is that between 0.61 and 0.51 per cent.

*Comparison of Microscopic Observation with Chemical Data*

Quantitative evaluations of the collagen and reticulum content were made from sections of livers stained with Gomori's reticulum and Masson's trichrome stain. These observations, expressed as one to four plus degrees of change (Tables I and II), were compared individually with the content of collagen as determined chemically. It was found that chemical values agreed far more closely with the evaluation based upon reticulum-stained, rather than trichrome-stained material. The latter sometimes revealed little collagen in

livers showing high chemical values, but in the same livers large amounts of argyrophilic fibers were revealed by the stain for reticulum. This suggests that the chemical method determines reticulum as well as collagen.

It is apparent from Table III that the total amount of fibrillar material as revealed by the reticulum stain parallels the chemical values rather closely.

TABLE III  
*Correlation between Chemical Determination and Microscopic Evaluation of Collagen Content by the Reticulum Stain\**

	++++	+++	++	+	0
Cirrhosis due to butter-yellow . .	0.67	0.47	0.40	0.29	0.25
Cirrhosis due to carbon tetra- chloride.....	1.14	0.68	0.58	0.59	

\* Numerical values expressed represent per cent collagen wet weight.

#### SUMMARY AND CONCLUSIONS

Quantitative determinations of collagen were carried out on rat livers showing cirrhosis due to *p*-dimethylaminoazobenzene and carbon tetrachloride.

A twofold increase in collagen content occurred in cirrhosis due to *p*-dimethylaminoazobenzene. The average total hepatic collagen as well as the per cent collagen content were doubled. The collagen content after 1½ months of normal diet had fallen to normal levels.

In cirrhosis due to carbon tetrachloride, the collagen content underwent about a fourfold increase. Partial reversal with significant decrease in collagen content occurred after stopping the carbon tetrachloride. The incomplete resorption of collagen in this group can be attributed to impaired hepatic regeneration following the prolonged administration of the compound.

The chemical values for collagen parallel the quantitative evaluations of collagen content, based on microscopic examination of liver sections stained for reticulum.

Deposition of collagen in hepatic cirrhosis is not necessarily an irreversible phenomenon.

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## EXPLANATION OF PLATES

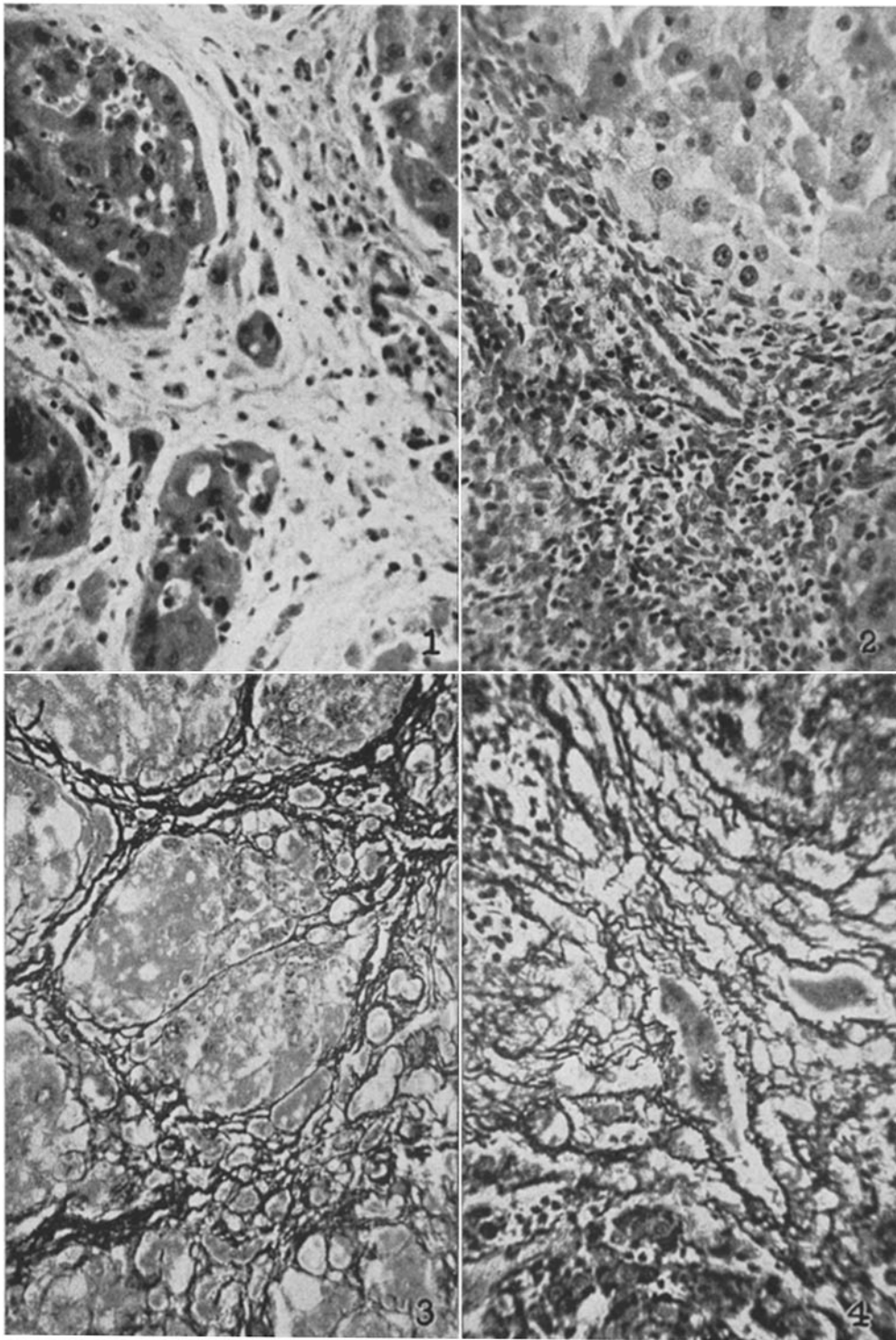
## PLATE 8

FIG. 1. Cirrhosis in a rat following  $3\frac{3}{4}$  months of exposure to carbon tetrachloride vapors. Wide fibrous septae separate pseudolobules of hepatic cells. Collagen content 1.10 per cent. Histological grading of cirrhosis + + + +.  $\times 300$ .

FIG. 2. Cirrhosis due to *p*-dimethylaminoazobenzene. The septae show greater cellularity than those of Fig. 1, with relatively less interstitial fibrous tissue. Collagen content 0.51 per cent. Histological grading + + +.  $\times 300$ .

FIG. 3. Pseudolobulation in cirrhosis following exposure to carbon tetrachloride vapors for  $3\frac{3}{4}$  months. There are large amounts of collagen and reticulum about irregular groups of hepatic cells. Gomori reticulum stain. Collagen content 1.32 per cent. Histological grading + + + +.  $\times 300$ .

FIG. 4. Abundant collagen and reticulum in a fibrous septum in cirrhosis due to *p*-dimethylaminoazobenzene. Gomori reticulum stain. Collagen content 0.47 per cent. Histological grading + + +.  $\times 300$ .

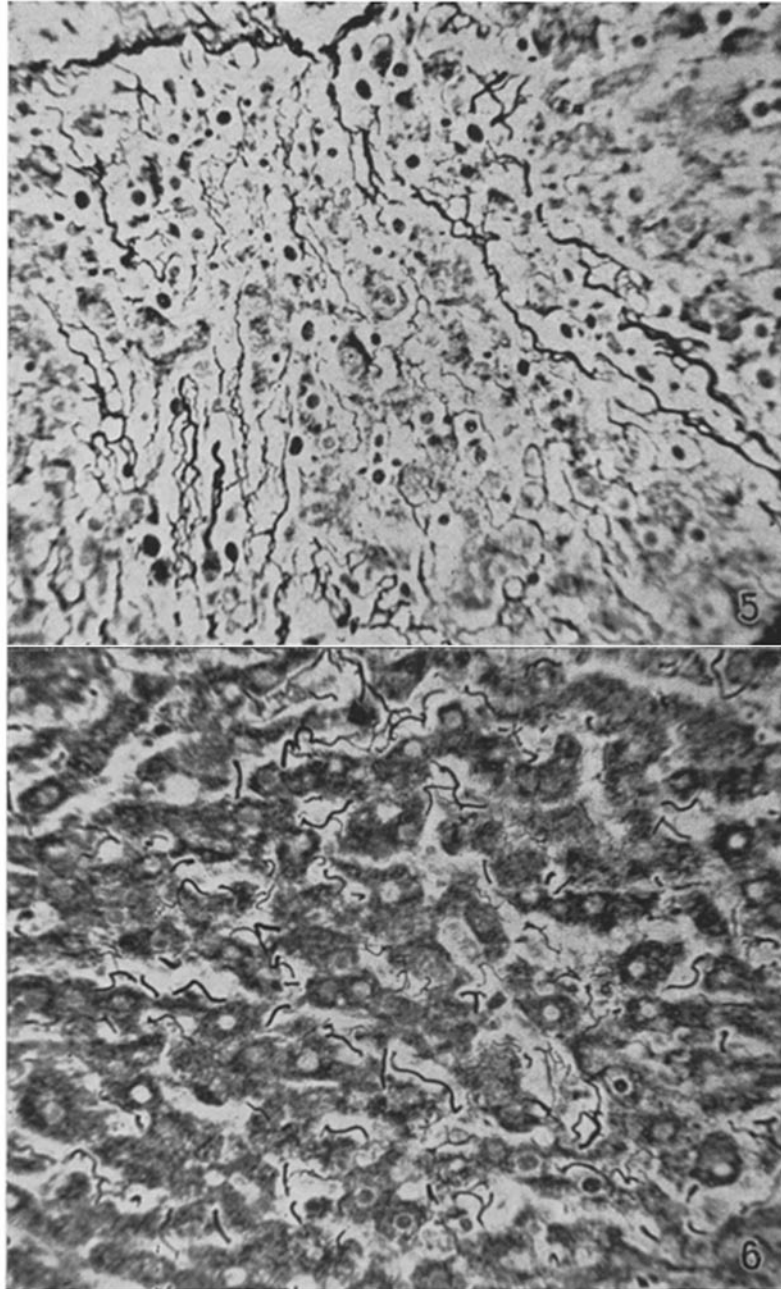


(Morrione: Collagen content in cirrhosis)

PLATE 9

FIG. 5. Scant residual argyrophilic reticulum following partial reversal of cirrhosis due to carbon tetrachloride. Gomori reticulum stain. Collagen content 0.39 per cent. Histological grading +.  $\times 300$ .

FIG. 6. Sparse argyrophilic reticulum in recovery from cirrhosis due to *p*-dimethylaminoazobenzene. Gomori reticulum stain. Collagen content 0.24 per cent. Histological grading +.  $\times 300$ .



(Morrione: Collagen content in cirrhosis)