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FURTHER OBSERVATIONS ON COLLAGEN IN REGENERATING LIVER OF THE RAT

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In a previous paper, experiments were described in which the collagen content of the rat's liver, regenerating after partial hepatectomy, was used as an indication of the growth of supporting tissue (Harkness, 1952*a*). Regeneration of collagen was found to lag far behind that of the liver as a whole, and was apparently incomplete 3 weeks after partial hepatectomy when the liver parenchyma is fully regrown. Collagen, however, was estimated chemically as a whole, and, consequently, the morphological significance of the changes was not clear. True collagen, judged by staining properties, is present in the peritoneal capsule of the liver, and in the walls and tissues surrounding the blood vessels and bile ducts. In addition, there is a network of reticulin in walls of the hepatic sinusoids. The available evidence indicates that this material is similar to collagen chemically (Bowes & Kenten, 1949), and the term collagen will be used to include it.

We thought that the regeneration of collagen at 3 weeks, although it did not reach the original level, might nevertheless be complete in the sense that it had reached an equilibrium state representing a somewhat altered liver structure. There are features of 3-week regenerated liver which lead one to suspect that it might be constructed with less than the normal proportion of collagen. Thus the lobes become more rounded with less relative surface area, so one might expect the capsular collagen to increase less than the weight of the liver. The fact that the lobes are more rounded should also make it unnecessary for the main vascular tree to expand as much proportionately as the weight. On the other hand, one might expect the sinusoids to reach their normal size relative to the parenchymal cells and hence to increase approximately in proportion to the weight of the liver. In order to clarify our previous results we have therefore separated the collagen in the liver into three fractions—capsular, vascular and parenchymal, the latter representing the collagen of the vessels most closely associated with the parenchyma—and estimated the collagen in these separate fractions at 3 and 6 weeks after partial hepatectomy. At 6 weeks the

increase of total collagen had still not caught up with the increase in weight. All fractions increased, but to different extents and in different proportions at 3 and 6 weeks. The greatest relative increase was in the capsular collagen.

A preliminary report of this work has already been published (Harkness & Harkness, 1953).

METHODS

Animals

Adult male albino rats of the local strain were used. They were partially hepatectomized by the method of Higgins & Anderson (1931) under ether anaesthesia. They were kept in individual cages in a thermostatically controlled room at 26° C for 1 week before operation and subsequently. They were allowed free access to water and food (M.R.C. diet 41, Parkes, 1946) at all times. The choice of rats for operation or control was made by reference to a table of random numbers. The animals were killed by a blow on the head.

Collagen estimation

Collagen was estimated by the method of Neuman & Logan (1950). The basis of this is estimation of the amino-acid hydroxyproline. Collagen contains 13·5% of hydroxyproline, which appears not to be present in other proteins except elastin. After preliminary extraction of soluble protein by 20% (w/v) urea solution, followed by water, the collagen is solubilized by autoclaving and then hydrolysed in 6 N-HCl; the hydroxyproline content of the hydrolysate is estimated. The method is therefore highly specific for collagen, since autoclaving eliminates most other protein including elastin. As our method involved some modification of the original, and certain parts of it required checking, it is briefly described.

The sample of liver was homogenized in a glass Potter & Elvehjem (1936) homogenizer in approximately ten times its volume of 20% (w/v) aqueous urea solution, in which it was left for 1 hr with occasional stirring; the solid was then centrifuged down (3000 rev/min) and washed by resuspension and centrifugation once with the same volume of urea solution and three times with distilled water. It was then extracted with alcohol: ether mixture (3:1, v/v) followed by ether, dried and autoclaved in a 12 ml. centrifuge tube with 5 ml. of distilled water for 6 hr at 30 Lb./sq. in. pressure. The volume was made to 10 ml. with distilled water and the tube heated for ½ hr on a boiling water-bath with occasional stirring to distribute the solubilized material evenly; the tube was cooled and the volume again adjusted to 10 ml. It was then centrifuged and the supernatant poured off and filtered through Whatman no. 1 paper. An aliquot, usually 7·5 ml., was removed and evaporated to dryness on a water-bath, and washed into a glass tube with 5 ml. of 6 N-HCl; the glass tube was sealed and autoclaved for 4 hr at 40 Lb./sq. in. It was then opened and a portion neutralized with an equal volume of 6 N-NaOH. Hydroxyproline was estimated on 1 ml. portions of the neutralized solution, the colour developed being measured in a Hilger absorptiometer with Ilford 635 spectral yellow green filters. The whole hydrolysate was not neutralized at once because it was found that the hydroxyproline content of the neutral solution fell off rapidly and was markedly diminished after a day.

In some cases a further modification was made in the method. Some of the larger samples of liver gave brown tinted hydrolysates. The colour was removed by shaking the hydrolysate with about 50 mg of charcoal which was immediately filtered off. This procedure was found not to affect the hydroxyproline content of the solution. We also checked two particular points in the estimation:

(1) *First centrifugation in urea solution.* We thought it possible that finely divided collagen might be lost in the supernatant since this was turbid. However, centrifugation of the supernatant at 6000 rev/min for 1 hr produced only very little sediment in which we detected no hydroxyproline.

(2) *Error caused by tyrosine in the final hydrolysate.* Neuman & Logan (1950) reported that tyrosine gave a colour development in the reaction equal to about 1% of that given by hydroxy-

proline. Only such large quantities of tyrosine as were very unlikely to be present would therefore affect the results. However, we felt it advisable to check this possibility. Tyrosine was estimated by the method of Medes (1932) on some hydrochloric acid hydrolysates which were taken to dryness on a water-bath and redissolved in 4N-H_2SO_4. Negligible quantities were found, and we therefore made no further estimations of tyrosine. The values for collagen given in this paper are not corrected for tyrosine error which would amount to less than 1% and are obtained from the hydroxyproline estimations by multiplying by 7·46, the factor given by Neuman & Logan (1950).

Fractionation of collagen in liver

The full fractionation procedure was only carried out on one lobe as the others were too small to be suitable. This lobe we have called the *anterior right lobe*. As we have been unable to find any standard nomenclature for all the lobes of the rat's liver it is necessary to explain briefly the one we have used. The *left* and *median lobes* are removed at operation. The residual lobes are the following:

(1) *The anterior right lobe* is the largest and most ventral, in shape similar to the median lobe.

(2) The next largest lobe is dorsal to the anterior right and because of its shape we have called it the *triangular lobe.*

(3) There are two smaller lobes lying in the lesser curvature of the stomach; these we have called the *anterior* (ventral) and *posterior* (dorsal) *caudate* lobes.

When the animal had been killed the residual lobes were subdivided as follows for analysis. The ligature left from the operation was removed with the small quantity of fibrous tissue surrounding it. Next the anterior caudate lobe was cut off at its base; we thought it advisable to analyse this lobe separately because it lies closest to the ligature and might be subjected to local inflammatory change which might cause excessive collagen deposition. Next the anterior right lobe was separated and subjected to the fractionation described below. The rest of the liver, i.e. the triangular lobe, posterior caudate and small amount of hepatic tissue joining them round the inferior vena cava, was subjected to part of the fractionation procedure.

The fractionation of the anterior right lobe was carried out as follows: first the capsule with as thin a layer as possible of underlying parenchyma was shaved off with a razor blade; then the rest of the lobe was pressed through a stainless steel plate 2·5 cm diameter and 2 mm thick, containing about 140 holes 1 mm in diameter, and fixed in the end of a hollow brass cylinder with a close-fitting plunger which could be screwed down tightly on to the plate. The larger vessels remain as an intact tree behind the plate while parenchyma is squeezed through, except for a small amount which remains in the holes in the plate attached to the vessels. Thus the lobe was separated into three fractions representing the capsule, the parenchyma, and the main vessels; the capsular and vascular fractions having with them some of the parenchymal fraction. In the final calculations we made a correction for this assuming that the weight of the vessels and capsule were negligible, the whole weight of the corresponding fractions being contaminating parenchyma. We assumed that this parenchyma had the same percentage of collagen as the parenchymal fraction as originally separated, subtracted this amount of collagen from the total for each fraction, and added it to the parenchymal fraction; we thus separated the collagen into three fractions which we have called 'parenchymal', 'capsular', and 'vascular', though the latter two could be more correctly described as representing capsular and vascular excess over parenchymal. The exact point of separation between the parenchymal and vascular fractions is difficult to define. Examination of stained sections of fixed parenchymal fraction indicated that the separation was mainly at the sublobular level. The sections showed fragments of parenchyma of variable size, some of which contained vessels corresponding in size to the interlobular vessels. The whole anterior caudate lobe was analysed without fractionation. The triangular and posterior caudate lobes were pressed through the plate and separated into a 'parenchymal' and 'residual' fraction, which corresponded to the combined 'vascular' and 'capsular' fractions of the anterior right lobe.

RESULTS

The weights of animals and principal parts of the liver are given in Table 1. The weights and collagen contents of individual lobes and fractions of lobes are given in Tables 2 and 3 and Figs 1 and 2. In order to compare changes in the different lobes and fractions of lobes of the liver we have expressed their weights and collagen contents as a percentage of the initial value, predicted from the weight of the liver removed at operation by multiplying this by the

TABLE 1. Body weights and liver weights.

	Normal animals		Partially hepatectomized animals	
	A	B	3 weeks	6 weeks
No. of rats	6	6	6	6
Body weight at start (g)	—	208±5	194±6	205±7
Body weight at death (g)	218±18	305±14	229±9	297±15
Weight of liver removed at operation (g)	5·49±0·57	7·35±0·55	5·48±0·35	5·44±0·38
Weight of regenerating lobes at death (g)	2·86±0·32	3·71±0·25	8·73±0·68	8·93±0·55
Estimated weight of regenerating lobes at operation (g)*	—	—	2·79±0·18	2·77±0·20

The estimate of variation is the standard error of the mean.

* Estimated by multiplying the weights of removed lobes by the mean ratio *lobes left:lobes removed* in control animals, i.e. 0·51±0·01.

A = killed at start of experiment; B = killed at 6 weeks. The description of the parts of the liver refers to the partially hepatectomized animals, and to the corresponding lobes in the normal animals.

TABLE 2. Fresh weight of separate lobes of the regenerating part of the liver as a percentage of the whole of this part and of estimated initial weight of the individual lobes.

	Control rats (% total)	Partially hepatectomized rats (% calc. initial weight)	
		3 weeks	6 weeks
Total	100	312±8	338±14
Anterior right lobe	45·4±0·9	323±12	351±20
Triangular and posterior caudate lobes	42·8±0·9	300±7	318±13
Anterior caudate lobe	11·9±0·3	315±21	356±23

The estimate of variation is the standard error of the mean.

appropriate ratio calculated from the control animals. These were originally divided into two groups, one killed when the partially hepatectomized animals were operated upon, and the other at the same times as the 6-week animals, to assess the changes which might take place over the 6-week period in the normal course of events without operation. In fact the relative weights of the different lobes and distribution of collagen within the lobes did not differ significantly in the two groups of control animals which were therefore combined for the purpose of predicting the initial values in the experimental animals.

It appears that the different lobes of the residual liver increase approximately equally in weight during regeneration. There are, however, some

TABLE 3. Collagen in the separate parts of the residual liver.

	Normal rats		Partially hepatectomized rats			
			3 weeks		6 weeks	
	Concn.	% total of lobe	Concn.	Total as % calc. initial	Concn.	Total as % calc. initial
Total all lobes	1·72±0·11	—	0·98±0·04	177±11	1·26±0·16	243±21
Anterior right lobe						
Total	1·57±0·11	100	0·99±0·04	206±11	1·18±0·19	267±29
Capsular	0·21±0·03	13·4±2·6	0·21±0·04	323±61	0·22±0·04	365±60
Vascular	0·67±0·06	42·8±4·0	0·33±0·02	159±15	0·51±0·09	257±37
Parenchymal	0·69±0·06	43·8±4·1	0·45±0·04	215±9	0·45±0·06	225±24
Triangular and posterior caudate lobe						
Total	1·89±0·11	100	0·95±0·10	151±15	1·37±0·16	226±18
Residual	1·11±0·08	48·7±3·5	0·49±0·05	134±17	0·95±0·18	269±38
Parenchymal	0·78±0·07	41·3±3·5	0·46±0·10	175±34	0·42±0·03	173±18
Anterior caudate lobe	1·71±0·13	—	1·04±0·11	187±17	1·28±0·04	264±14

The estimate of variation is the standard error of the mean. The columns headed 'concn.' give the quantity of collagen (mg) per g fresh wt. of the italicized parts of the liver in the left-hand column, e.g. *anterior right lobe.*

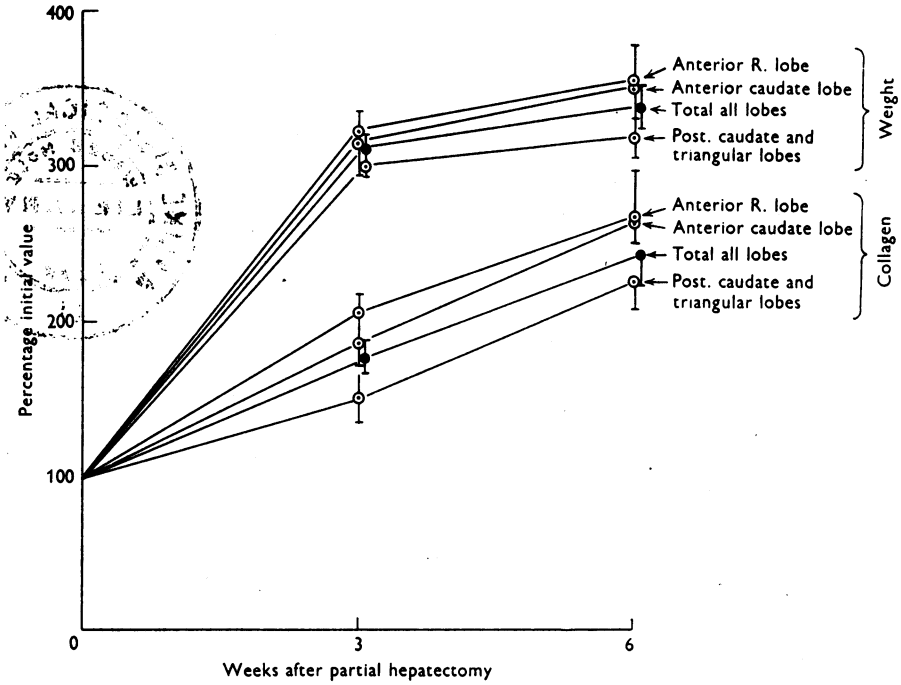

Fig. 1. Wet weight and total collagen of separate liver lobes as percentage of calculated value at time of operation. (*Note.* The vertical lines extend from + to − the standard error of the mean.)

differences; though the anterior right and caudate lobes increase equally, the increase is consistently less in the more posterior triangular and posterior caudate lobes.

As regards collagen, the results at 3 weeks are similar to those previously reported (Harkness, 1952*a*); collagen only rises to about two-thirds the original total by this time. Between 3 and 6 weeks there is a further increase, and the total reaches nearly the original. The weight of the liver has, however, risen well above the original, and is still therefore almost as far ahead of the collagen

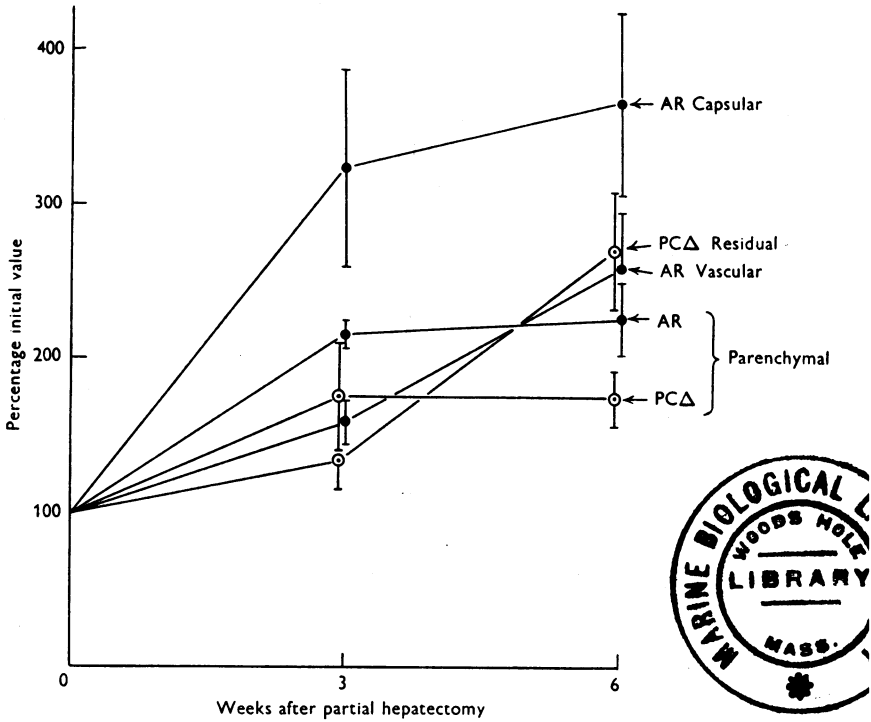

Fig. 2. Collagen of separate fractions of anterior right (AR) and posterior caudate and triangular (PCΔ) lobes. (*Note.* The vertical lines extend from + to − the standard error of the mean.)

in growth as at 3 weeks. It appears that the 3 weeks' state is not one of equilibrium, and that, if the relation of collagen to liver weight ever returns to normal after partial hepatectomy, it is only after a relatively long time. The increase of total collagen of the individual lobes shows the same difference between lobes as appeared in weight. In the anterior right lobe in normal animals only about one-sixth of the total collagen was in the capsular fraction, the remainder being divided equally between the vascular and parenchymal fractions. The combined posterior caudate and triangular lobes had a similar fraction in the parenchyma, the remaining collagen in the latter lobe corresponding to the combined capsular and vascular fractions of the anterior

right lobe. Contrary to expectation the greatest proportional increase throughout regeneration was in the capsular fraction. In the anterior right lobe at 3 weeks the parenchymal fraction had increased more than the vascular, but between 3 and 6 weeks it increased very little more while the vascular fraction increased considerably. The capsular fraction is relatively small and change in the residual non-parenchymal fraction of the posterior caudate and triangular lobes will reflect mainly change in the vascular fraction. The residual non-parenchymal fraction of the posterior caudate and triangular lobes shows similar change to the vascular fraction of the right anterior lobe.

DISCUSSION

In these experiments we have used the wet weight of the liver as an index of the growth of the organ for comparison with change in collagen content. This could not be done without qualification, if we had been investigating the early stages of regeneration when considerable changes in the composition of the organ take place. It appears from available evidence, however, that the composition of the liver returns to normal before the times we have investigated, i.e. 3 and 6 weeks postoperatively. The results of Bogetti & Mazzocco (1939) indicate that the total solid content of the liver comes back to normal about the 8th day after partial hepatectomy, though perhaps one should not place too much reliance on this, because their results show relatively enormous and unexplained fluctuations between operation and the 8th day. Gurd, Vars & Ravdin (1948) give average total solid figures of 29·9 and 30·5 g/100 g wet weight respectively for controls and animals killed 14 days after partial hepatectomy. Crandall & Drabkin (1946) give a figure for 14-day regenerating liver which differs slightly but significantly in the same sense, i.e. greater solid content, from liver removed at operation. However, it has been pointed out (Harkness, 1952*b*) that the handling of the lobes which is inevitable in their removal at operation may cause minor oedema which could give rise to a small difference in the solid content in the sense found. It is of interest that Crandall & Drabkin, in the same paper (1946), give a figure for the solid content of normal rat liver which is higher than that for liver removed at operation, and nearer to their figure for 14-day regenerating liver, from which it does not differ significantly (*t* test, $P > 0{\cdot}05$, Fisher, 1946). The individual components of liver appear generally to return to normal concentration between the 7th and 14th postoperative days approximately. Gurd *et al.* (1948) found practically no difference in total protein content of normal and 14-day regenerating liver; the concentration of collagen in liver is so low that such changes in its concentration as occur do not affect the total protein content appreciably. Total lipid (Bogetti & Mazzocco, 1939; Gurd *et al.*, 1948), neutral fat, total phospholipid, free and ester cholesterol concentrations (Ludewig, Minor & Hortenstine, 1939), are practically normal by the end of the first week after

partial hepatectomy. Glycogen is back to normal concentrations by the end of the first postoperative week according to Gurd *et al.* (1948), though Stone (1935) reports it as still somewhat below normal as late as the 28th day. Novikoff & Potter (1948) report that ribonucleic acid (PNA), after an initial rise, falls back to normal concentration about the end of the first week. Johnson & Albert (1952) record PNA concentration as still above normal at 14 days, while Drabkin (1947) records it somewhat below normal at this time. The concentration of desoxyribonucleic acid (DNA) is recorded as somewhat above normal at 14 days by Johnson & Albert (1952), and considerably above by Drabkin (1947). This raised DNA concentration may represent an increase in the population of cells of the macrophage type, reported by Abercrombie & Harkness (1951) as present on the 7th but not on the 21st day. It does not seem worthwhile to review here the whole literature on enzymes in regenerating liver; it appears that most of the systems investigated return to nearly normal activity within about a week, though there are exceptions. Thus it appears that the composition of the rat's liver regenerating after partial hepatectomy returns in most respects to normal within 2 weeks.

It seems clear from our results that the low degree of regeneration of collagen previously found at 3 weeks (Harkness, 1952*a*), does not represent an equilibrium state of liver structure altered in accordance with the modified shape of the regenerated liver. Though the change of shape of the liver must have reduced the area of the peritoneal capsule relative to the weight, the capsule showed a greater relative increase than any other fraction of the collagen. A possible explanation of this is a mild inflammatory reaction resulting from the operation. Of the other two fractions the parenchymal regenerated faster than the vascular. This was expected since the parenchymal contains the reticulin and is closely associated with cells which return very nearly to their normal number and size (Abercrombie & Harkness, 1951) by 3 weeks. Nevertheless, this parenchymal fraction did not return to normal; the quantity of collagen in it per unit weight of lobe is still below normal both at 3 and 6 weeks. One cannot tell whether this abnormality reflects change in the structure of the sinusoids or of the small interlobular and other vessels in the fraction or both.

It appears that different factors determine the formation of collagen in the different fractions. There were also some differences between lobes. It is known that the streams of blood entering the portal veins do not mix completely in normal animals, that from the spleen going mainly to the left while that from the mesenteric vein goes to the right of the liver (see Hahn, Donald & Grier, 1945). A difference in the composition of the blood reaching the different lobes could explain our results, but this would have to be a dorso-ventral difference and this does not appear to have been observed.

SUMMARY

1. Increase of collagen in regenerating liver has been investigated in rats 3 and 6 weeks after partial hepatectomy.

2. The collagen content of the liver still lagged behind increase in fresh weight of liver even at 6 weeks, though it had drawn somewhat nearer to it than at 3 weeks.

3. A procedure for separating different fractions of liver collagen was used. These fractions behaved differently during regeneration. The greatest increase in collagen was in the capsular fraction.

4. The separate lobes of the liver increased approximately equally in weight and collagen content, though there were some small differences.

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