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COLLAGEN IN REGENERATING LIVER OF THE RAT

By R. D. HARKNESS

From the Department of Physiology, University College, London

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There is a considerable amount of information available about biochemical and histological changes in the liver during regeneration after partial hepatectomy. Almost the whole of this information is, however, about changes in the hepatic parenchymal cells which make the greater part of the liver; very little is known specifically about changes in the other constituents. This is perhaps not surprising because the changes in hepatic cells are so very conspicuous. It is clear, however, that the rapid growth of hepatic parenchymal cells is only a part, if a major one, of the more general morphogenetic problem of the reconstitution of the liver as a whole organ. As an approach to this problem the collagen content of regenerating liver, as an index of growth of supporting tissue, has been taken for study. The results show that formation of new collagen lags considerably behind the formation of new parenchymal tissue. A preliminary account of this work has already been published (Harkness, 1950).

METHODS

The rats used were male and female albinos weighing 100–300 g. At operation the median and left lateral lobes of the liver were removed aseptically under ether anaesthesia (Higgins & Anderson, 1931). The animals were taken in batches of the same sex and approximately the same weight. In general, equal numbers from each batch were chosen for operation randomly (by reference to a table of random numbers) and killed 1, 2, 3, 7 or 21 days after operation, or were chosen to serve as normal controls. In some smaller batches 1- and 21-day animals were omitted. The animals were kept in a room at 26–27° C from a week before the operation until death, and allowed free access to water and food (M.R.C. diet 41; Parkes, 1946) at all times.

Estimation of collagen

Initially the choice seemed to be between two methods, that of Lowry, Gilligan & Katersky (1941), or that of Spencer, Morgulis & Wilder (1937). The former was chosen after both had been tried in the modified forms described below. Animals were killed by breaking the neck. Liver for analysis was removed rapidly, blotted, and chopped up into small pieces of about 100 mg with fine scissors. The fragments were mixed in a beaker to secure a reasonably homogeneous mass for sampling. This technique was used in the absence of a homogenizer which was capable of cutting up the connective tissues of liver and distributing them evenly in a suspension.

Method of Lowry, Gilligan & Katersky (1941). Samples of about 500 mg of chopped liver were weighed and ground with about ½ ml. of distilled water in a small Potter-Elvehjem homogenizer. The ground up tissue was washed out with N/10-NaOH into a centrifuge tube, and its volume made up to about 12 ml. with the same solution. It was then shaken thoroughly and allowed to

stand with occasional further shakings for 24 hr at room temperature (preliminary extraction). This extraction removes most proteins other than collagen and elastin. It was then centrifuged at 3000 rev/min for 1 hr. The supernatant was drawn off and the solid resuspended in N/10-NaOH in which it was left for 2 hr with occasional shaking (second extraction). The solid was then centrifuged down again and suspended in distilled water. A drop of 0·1% phenol red was added and the pH was adjusted to 7–7·5 by adding dilute hydrochloric acid (about N/100), sufficient time being allowed for all the alkali to diffuse out of the suspended material. It was then centrifuged and resuspended in a mixture of 3 vol. 95% ethanol and 1 vol. of ether for about 10 min, centrifuged again, resuspended in ether for 10 min and recentrifuged. The supernatant ether was then drawn off and the sample allowed to dry at room temperature. Collagen was then estimated from the amount of N made soluble by autoclaving (Abercrombie & Johnson, 1946) instead of from loss of weight after autoclaving as in the original method. About 5 ml. of distilled water was added. The tube containing the sample was then put in a beaker, covered with a Petri dish lid, and autoclaved for 6 hr at 30 lb./sq.in. pressure. The volume of fluid was made up to about 10 ml. with distilled water, and the sample was placed in a boiling water-bath for 0·5–1 hr with occasional stirring to get the solubilized material evenly distributed in solution. It was then cooled, made up to exactly 10 ml. and centrifuged. A 7·5 ml. aliquot was taken for digestion overnight with 0·5 ml. of concentrated sulphuric acid to which was added 250 mg of a mixture of K_2SO_4 and sodium selenate (25 mg sodium selenate to 10 g K_2SO_4). Since the amount of ammonia formed was too small to be estimated easily by the usual micro-Kjeldahl distillation, it was estimated colorimetrically by nesslerization. A photoelectric colorimeter of the type described by King (1946) was used.

In some of the samples of regenerating liver, mainly those taken at 24 hr, the preliminary NaOH extract was very viscous, so that the insoluble material fell more slowly than usual during centrifugation and had not gone down to the usual small compact plug after an hour. When this happened some of the supernatant was drawn off and replaced by N/10-NaOH. The tube was shaken and immediately centrifuged again, before the second extraction with N/10-NaOH.

Method of Spencer, Morgulis & Wilder (1937). In the original method the liver was dried with acetone and powdered. Samples of the powder were autoclaved with water at 20 lb./sq.in. pressure for 2 hr. The liberated gelatin was precipitated by tannic acid, and the amount estimated by determination of nitrogen.

This method was modified first by omission of the drying in acetone. The same modification has been used by Lightfoot & Coolidge (1948). The samples of liver were homogenized and placed in a boiling water-bath for 10–15 min with 5 to 10 times their weight of water. They were kept in the refrigerator overnight, and autoclaved the next day. After this they were placed on a boiling water-bath for 0·5 hr with occasional stirring to distribute the solubilized material evenly, and then filtered through Whatman No. 1 filter-paper. Samples of the clear fluid obtained were then treated either with an equal volume of 10% trichloroacetic acid to precipitate material other than gelatin (Janota, 1943), or acidified and treated with an equal volume of 5% tannic acid (B.P.) as in the original method. In each case the precipitated material was centrifuged down, and nitrogen in it estimated by the micro-Kjeldahl procedure. After digestion by the same procedure as used for the method of Lowry *et al.* the ammonia was distilled into 1% boric acid with methyl red-bromcresol green indicator (Conway, 1947), and titrated with standard hydrochloric acid.

RESULTS

Assessment of methods

Effect of autoclaving time. Lowry *et al.* recommended a time of 4 hr at 50 lb./sq.in. pressure or 6 hr at 25 lb. Spencer *et al.* used 2 hr and 15–20 lb. pressure. In this experiment samples were autoclaved for 1, 2, 3 and 5 hr at 30 lb. The results are given in Fig. 1.

In the Spencer method the solubilized N was much greater than in the Lowry method. The N precipitable by trichloroacetic acid rose to a maximum at 2–3 hr then fell. The N precipitable by tannic acid rose throughout the 5 hr. It was found that if the supernatant from the trichloroacetic precipitation was precipitated with tannic acid, the amount of nitrogen in the precipitate, when added to that in the previous trichloroacetic precipitate, was equal to that obtained by tannic acid precipitation alone in the original solution. In one experiment tannic acid precipitation of a sample gave 0·66 mg more N than trichloroacetic precipitation, while tannic acid precipitation of the trichloroacetic supernatant gave 0·64 mg N. In another experiment the

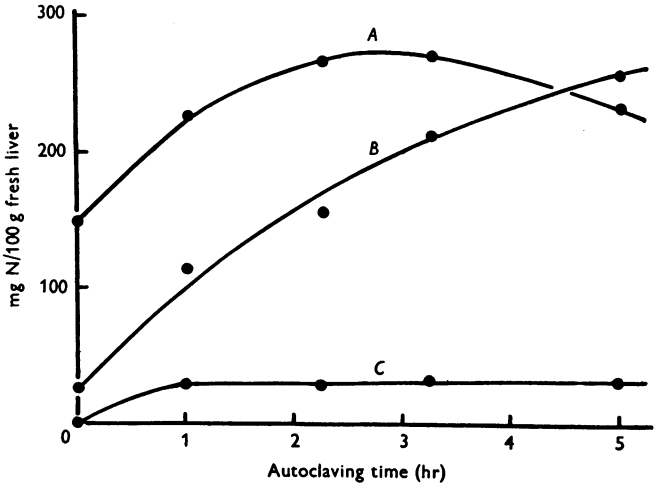

Fig. 1. Effect of autoclaving time, at 30 lb./sq.in. pressure, on amount of N brought into solution. Curves *A* and *B*, method of Spencer *et al.* (1937); curve *A*, trichloroacetic precipitable N; curve *B*, tannic acid precipitable N; curve *C*, method of Lowry *et al.* (1941).

corresponding figures were 0·89 and 0·91 mg N. In other words, it is probable that the material precipitated by trichloroacetic acid, which is not gelatin (Janota, 1943), is also precipitated by tannic acid. Thus it seems that the method is wrong at least by the amount of the trichloroacetic acid precipitable N. It seems probable that a considerable amount of the other tannic acid precipitable material is also not gelatin, for it increases continuously up to 5 hr autoclaving. Indeed, Fig. 1 indicates that it would increase still further if the time were increased. Collagen should be completely solubilized by this time and probably reticulin also if liver reticulin behaves like that of spleen and fatty tissue (see Bowes & Kenten, 1949). It was thought that these high results might be due to omission of the acetone-drying process. However, liver dried in this way gave almost the same results as above when autoclaved at 30 lb for 5 hr.

In the Lowry method the solubilized nitrogen rose almost to its maximum at 1 hr. None of the solubilized material was precipitable with trichloroacetic acid.

As a result of these experiments the method of Lowry *et al.* was chosen and checked further as follows.

Effect of variation in conditions of extraction with NaOH (*Method of Lowry, Gilligan & Katersky*). The effect of varying the conditions of extraction with N/10-NaOH was investigated in order to find out whether there was any loss of collagen. It is during the extraction with NaOH that loss is most likely to occur.

First, the effect of carrying out the preliminary extraction at 0° C instead of at room temperature and the effect of prolonging it, at room temperature, to 48 hr were investigated. Samples from a single normal liver were used. Five samples estimated by the standard method (24 hr at room temperature) gave a mean of 34·5 mg collagen N per 100 g fresh liver, S.E. $\pm$ 1·7. The corresponding figure for four samples extracted for 24 hr at 0° C was 33·1 $\pm$ 0·6, and for four samples extracted for 48 hr at room temperature was 34·4 $\pm$ 3·3.

Secondly, the effect of repeating the preliminary extraction was investigated. At the end of 24 hr the sample was centrifuged, and the solid resuspended in N/10-NaOH for a further 24 hr at room temperature. In one experiment four samples from normal liver gave a figure of 28·4 $\pm$ 1·0 mg collagen N per 100 g fresh weight by the standard method; four samples in which the extraction was repeated gave 21·3 $\pm$ 1·9 mg collagen N per 100 g fresh weight. Corresponding figures for a second experiment were 33·3 $\pm$ 1·0 and 28·4 $\pm$ 1·8 (three samples).

From these experiments it was concluded that there is danger of loss of collagen during extraction on N/10-NaOH but that the losses must be normally small.

Nature of the material solubilized. Lowry *et al.* (1941) checked by Millon's test that their solubilized material after autoclaving contained no detectable tyrosine. It was thought advisable to extend this check. With this object, some of the material estimated as collagen was hydrolysed with 6 N-HCl for 24 hr at 100° C. The amino-acid composition of the hydrolysate was then investigated by filter-paper chromatography. The acid hydrolysate was neutralized, desalted in an apparatus similar in principle to that of Consden, Gordon & Martin (1947), and run on a 20 $\times$ 20 cm square of Whatman No. 1 paper first in phenol ammonia, then in collidine in the apparatus described by Datta, Dent & Harris (1950). The amino-acid pattern was developed by spraying with 0·1% ninhydrin in butanol. This pattern (Fig. 2) was, within the quantitative limits of the method, the same as one would expect from published analyses of the amino-acid composition of collagen (see Bowes & Kenten, 1949). The high glycine, proline and hydroxyproline content and

low tyrosine content found are particularly characteristic of collagen and allied substances. The pattern of spots was compared with a sample of gelatin (B.P.) hydrolysed and otherwise treated in the same way. The two patterns were practically identical when samples containing the same quantity of nitrogen were compared. When the comparison was made on a weight basis, however, about twice the weight of 'collagen' was needed to match a given weight of gelatin. This was almost certainly because the 'collagen' contained silicious material which probably came from the homogenizer. It was identified tentatively as silicious since it resisted boiling in concentrated sulphuric

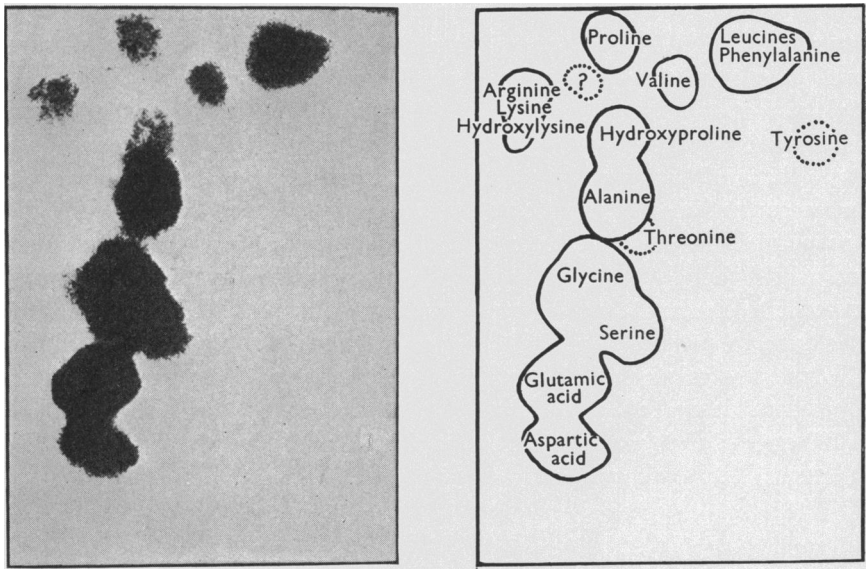

Fig. 2. Paper chromatogram of the amino-acids in an acid hydrolysate (37·5 μg N) of the material estimated as collagen by the method of Lowry *et al.* (1941). Phenylalanine and the leucines are not separated since diethylamine was not used in the collidine run. The dotted lines on the key represent spots which were just visible on the original chromatogram, but did not appear in the reproduction.

acid overnight. The possibility of errors arising from this source seems to argue in favour of the use of the amount of N solubilized by autoclaving, rather than the loss of weight of the material, as a measure of collagen.

The chromatographic evidence showed that the material estimated as collagen was this substance, at least for the most part. In order to estimate roughly the amount of contamination which could be detected, the collagen hydrolysate was run as a paper chromatogram with various amounts of similar hydrolysate of whole liver. 'Collagen' hydrolysate containing 25 μg of N gave no tyrosine spot on the paper, whereas hydrolysate containing

37·5 μg of N gave a just faintly visible spot. This amount of tyrosine is less than 5 μg, which gives a definite spot on the paper; that is, a quantity of the order one would expect from published analyses to find in the amount of collagen hydrolysate used. 'Collagen' hydrolysate containing 25 μg of N plus liver hydrolysate containing 5 μg of N gave, however, a clearly detectable tyrosine spot. It would seem then that on a N basis 10% contamination with whole liver protein would be detectable by the chromatographic method. The slight changes which must have occurred in the relative proportions of other amino-acids after addition of hydrolysate were not detectable.

Distribution of collagen in the liver. The above experiments seemed to show that the method of Lowry *et al.* was reliable and really did estimate collagen; nevertheless, duplicates were never good. For example, in a set of twenty pairs of estimations on normal livers the mean coefficient of variation was 11·3 %. This was put down to the difficulties of sampling, it being presumed that the collagen content varied in different parts of the liver, being greater at the base of the liver lobes than at the periphery. This appears to be so macroscopically, and in sections stained by Mallory's or Pasini's method. It was found difficult to mix the liver for sampling even after it had been minced, and it was thought that the bad duplicates were due to inadequate mixing. In order to confirm that this explanation was the most likely, an experiment was done to determine chemically the distribution of collagen in the liver. A lobule was taken and the collagen in samples from the base, middle and edge were estimated separately. The results were: base, 56 mg collagen N/100 g wet weight; middle, 28 mg collagen N/100 g wet weight; edge, 25 mg collagen N/100 g wet weight.

Collagen in regenerating liver

The concentration of collagen N in the regenerating liver at different times after operation is given in Table 1 and Fig. 3. It fell to a minimum at 48 hr post-operatively and then rose, but was still below normal at 21 days.

The total quantity of collagen N in the regenerating liver is given as a percentage of the total present preoperatively in Table 1, and Fig. 4, together with the fresh weight of the liver. The total weight of the liver pre-operatively was estimated from the weight of the liver lobes removed, and the ratio between the weight of these lobes and the total liver in the control animals. The total collagen N in the liver preoperatively was calculated from the estimated liver weight, and average concentration of collagen N in the livers of control animals. It is clear that collagen is formed in regenerating liver very much more slowly than parenchymal tissue; the whole course of the collagen curve falls below the curve of liver weight. It appears that new collagen begins to be formed between the 2nd and 3rd day after operation. The total on the first and second day is not significantly different from the

TABLE 1

	Normal rats	Experimental rats. Time after operation (days) 1	2	3	7	21
Total no. of rats	10	8	9	8	9	6
Males	3	3	3	3	2	0
Females	7	5	6	5	7	6
Body wt. (g)	156±13	166±15	150±11	147±14	157±13	140±13
Loss or gain of wt. after operation (g)	—	−12±1	−9±1	−4±3	−2±2	+21±4
Liver weight:						
Estimated total at time of operation (g)	6·16±0·24	6·50±0·49	6·06±0·33	5·82±0·22	6·16±0·33	5·78±0·42
Lobes removed at operation (g)	4·05±0·16	4·27±0·32	3·98±0·22	3·83±0·14	4·04±0·16	3·79±0·13
Liver weight at death (g)	2·11±0·09	2·96±0·22	3·54±0·17	3·91±0·20	4·55±0·17	5·71±0·27
Liver weight at death as % total at time of operation	34·4±0·5	45·5±1·3	58·7±2·0	66·6±1·9	73·1±3·1	100·2±4·8
Collagen N:						
Regenerating lobes (mg/100 g fresh wt.)	24·4±1·4	20·9±1·3	14·8±1·0	15·6±1·1	16·2±1·0	17·3±1·0
Lobes removed at operation (mg/100 g fresh wt.)	26·9±1·7	—	—	—	—	—
Total liver collagen at death as % of total at time of operation	32·3±0·8	36·6±2·6	32·2±2·0	40·5±3·3	45·8±3·8	66·4±5·5

The estimate of variation is in each case the standard error of the mean. Body weight is weight of normal rats at death, of partially hepatectomized rats immediately before operation. The description of parts of liver under 'Liver weight' and 'Collagen N' refers to experimental animals; the data on normal rats refer to the corresponding lobes.

Note. Data on four other rats were obtained, one at 2 days, two at 3 days and one at 21 days. The collagen estimations gave abnormally high results and were rejected. The general conclusions are not affected if they are retained. In the case of the 21-day rat there was almost certainly a technical error which was suspected also in the other three rats, which formed a consecutive group together in the first experimental series.

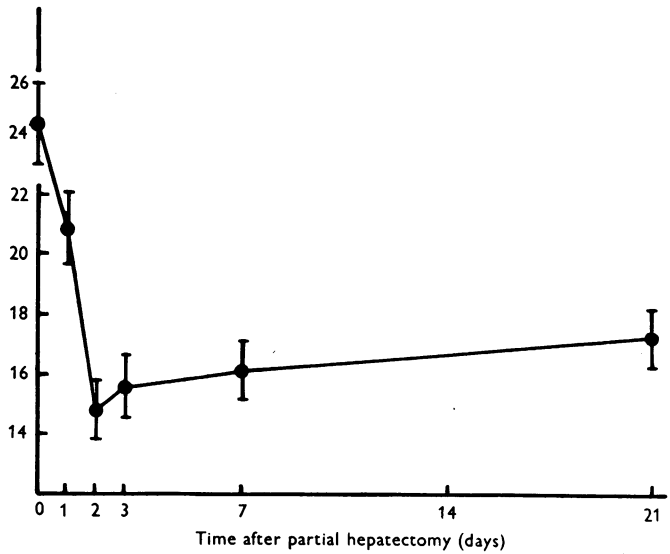

Fig. 3. Collagen N (mg/100 g fresh liver) in the liver at different times after partial hepatectomy.

total initially. The total on the 3rd day is significantly above that of the normal ($0{\cdot}02 > P > 0{\cdot}01$) or the normal and the first 2 days combined ($P = 0{\cdot}02$), by 'Student's' t test. It is doubtful, however, whether the information available is sufficient to determine when new collagen starts to be formed with certainty.

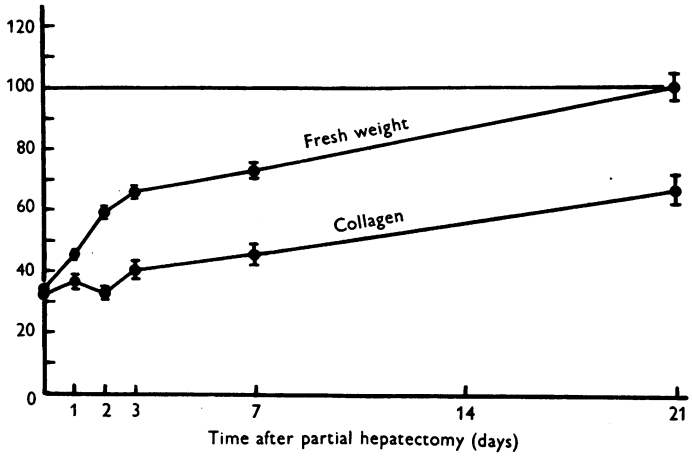

Fig. 4. Total fresh weight, and total collagen, in the liver as a percentage of the total preoperatively, at different times after partial hepatectomy.

DISCUSSION

The results of this investigation show that growth of collagen in regenerating liver lags considerably behind growth of the whole liver in weight. However, this latter is the sum of a complex series of changes in its various specific constituents and it is of more interest to compare the growth of these individually with that of collagen. The bulk of the liver is made up of the parenchymal cells. Under the experimental conditions used for the present investigation, these increase in size for about 24 hr after operation (Brues, Drury & Brues, 1936) and then begin to divide rapidly (Brues & Marble, 1937). The maximum rate of division appears to occur at about this time, and to diminish thereafter, while the parenchymal cells return to their normal size. However, growth of cells other than parenchymal appears to lag initially behind that of parenchymal cells. Thus it has been reported (Abercrombie & Harkness, 1951) that there is negligible cell division among any cells other than parenchymal at 24 hr but a considerable outburst at 48 hr. At this time active mitosis was found in the littoral cells of the hepatic sinusoids (Kupffer and so-called undifferentiated lining cells), in bile duct epithelium, in blood vessels, and in mesenchymal cells both in and under the peritoneal coat of the liver. However, it would appear that the growth of these other cells also precedes that of collagen since the latter did not appear in the present experiments to have increased at all in total at 48 hr. Abercrombie & Harkness

(1951) found that the number of littoral cells had reached normal by the 7th day after operation. Regeneration of these cells therefore precedes that of collagen along its whole time course. Though these workers were unable to follow the whole time course of regeneration of bile ducts and blood vessels quantitatively, it appeared that at 21 days their regeneration fell below normal in a manner comparable to that of collagen. Apart from this slender link there is no evidence at the moment to connect the growth of collagen with that of any particular group of cells in the liver. Nothing is known about the growth of fibroblasts in regenerating liver. However, it is perhaps relevant to note here that these cells have been found to show a greatly increased tendency to migrate in tissue culture of explants of 2-day regenerating liver though explants from 1-day liver did not show any such tendency (Abercrombie & Harkness, 1951). This change in the properties of liver fibroblasts in tissue culture therefore appears also to precede the deposition of collagen in regenerating liver.

Though growth of collagen cannot be connected yet with growth of any particular cellular type in the liver, some of the chemical constituents of this organ show a similar initial lag. Harkness (1949) found the volume of blood in the small vessels to do this, and Hawkins & Walker (1951) found the same for DOPA decarboxylase. Unfortunately, the location of this enzyme in the liver does not appear to be known for certain yet.

Thus for the moment the data given here on growth of collagen in regenerating liver must remain as isolated facts. As a first step towards their interpretation it would be of great interest to find out how the different fractions of liver collagen, in blood vessels, capsule, etc. regenerate. The investigation is being continued along these lines.

SUMMARY

1. The method of Lowry *et al.* (1941) for estimation of collagen has been investigated, and a modification has been used for estimation of collagen in the regenerating liver of the rat.

2. The results show that formation of new collagen in regenerating liver lags considerably behind formation of new parenchymal tissue. Even 21 days after partial hepatectomy the total amount in the liver was still below the total preoperatively.

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