

RETICULIN AND COLLAGEN. BY M. CHRISTINE TEBB.

(From the Physiological Laboratory, King's College, London.)

THE fibres of reticular or retiform tissue are anatomically continuous with those of areolar tissue, and are not distinguishable from them on microscopic examination¹. Mall², however, stated that no gelatin was obtainable from the fibres of reticular tissue, and that they were, therefore, chemically different from ordinary white fibres. The fact that gelatin is obtainable from reticular tissue was first demonstrated by R. A. Young³, and subsequently by Siegfried⁴. Siegfried, however, confirmed Mall's view that the fibres contained something special, and separated from them a material which he called reticulin. If such a chemical substance does exist, the point is by no means proved that reticular fibres are different from white connective tissue fibres; it is at least equally possible that reticulin is not specially characteristic of reticular fibres, but is also present in all white connective tissue fibres.

Accordingly, at Prof. Halliburton's suggestion, I sought by following Siegfried's directions as closely as possible to separate reticulin from a typical form of connective tissue, namely, tendon. My failure to obtain this substance from tendon led me next to repeat Siegfried's experiments on the tissue with which he himself worked, namely, the mucous membrane of the intestine. Here, again, I failed to obtain reticulin; the only organic substance present in appreciable quantity in the connective tissue basis is collagen. My experiments have led me to believe that Siegfried's reticulin is an artifact, and is mainly produced by the effect of ether and alcohol on collagen. These reagents render collagen very insoluble and hinder greatly its subsequent conversion by hot water into gelatin.

¹ Schäfer. *Quain's Anatomy*. 10th Ed. i. p. 239. 1891.

² *Anat. Anz.* III. No. 14, 1888; *Abhandl. d. math. phys. Cl. d. k. Sächs. Gesellsch. d. Wissensch.* XIV. No. 3, 1887; XVII. No. 4, 1891.

³ *This Journal*, XIII. p. 332. 1892.

⁴ "Ueber die chemischen Eigenschaften des reticulirten Gewebes," *Habilitationsschrift*. Leipzig. 1892.

Such being the main results of the investigation, I propose to support my statements by recording a number of typical experiments. It will, however, be first necessary to describe those of Siegfried rather more in detail. He used the *mucosa* of pig's intestine, which he always obtained from a butcher whose assistant had been instructed in separating it from the *submucosa*. The *mucosa* of 8—17 pigs was placed in about 40 litres of water at 37° C. with 25—30 grms. of Parke Davis and Co.'s pancreatin, 50 grms. of sodium bicarbonate, and some sodium carbonate and thymol or chloroform. After 48 hours the tissue was well washed with water, collected by a centrifugal machine and freed from water by alcohol. It was then extracted with ether in a Soxhlet's apparatus for several days. The tissue was again digested with pancreatin with less water for a further period of 48 hours. After washing with water and drying with alcohol and ether, Siegfried describes the reticular tissue as being in grey strands, which swelled in water to porous membranes having the structure of the original tissue, and he says that the microscope showed pure reticular tissue free from connective tissue fibres and lymph cells. Siegfried's principal statements concerning reticular tissue prepared in the manner described are as follows:

(1) If it is boiled for half-an-hour, it loses its structure and is transformed partly into a loose powdery substance, which is the author's reticulin, and partly into gelatin.

(2) Almost all the gelatin-yielding material can be extracted from the tissue by boiling for 20 minutes; and from this it is inferred that the material converted into gelatin is something other than collagen, which cannot be converted into gelatin in so short a time.

(3) The percentage composition of the so-called reticulin is given as: C, 52·88; H, 6·97; N, 15·63; S, 1·88; P, 0·34; ash, 2·27. It differs from collagen in containing phosphorus, in yielding no gelatin on boiling, and in yielding little or no glutaminic acid on being boiled for 72 hours with hydrochloric acid.

I now pass to the description of my own experiments, and will first take those in which I sought for reticulin in fibrous tissue, with negative results.

Exp. I. Three-quarters of a pound of tendon (ox) was superficially freed from fat, cut into small pieces and left in water over night; after which it was finely divided and soaked in about 7 litres of saturated lime-water, occasionally renewed, with some thymol, for six weeks. It was then washed in a large quantity of water and for three minutes in 0·2% acetic acid (which

did not more than neutralize the adherent lime), and finally in more water. It was then digested in a 2% solution of Benger's *liquor pancreaticus*¹ (to which 0.5% sodium carbonate and some chloroform were added) for 48 hours at a temperature of 37° C. The digestive fluid was then renewed, and the tendon was digested for a further period of 96 hours. The volume of fluid used in each case was about 15 litres. During digestion the quantity of tendon apparently diminished, pointing to the inference that some of the collagen itself had suffered digestion in spite of its never having undergone any preliminary treatment with acid² or boiling water. The tendon was ground in a mortar and digested finally for 2 days. It was then well washed and boiled in water for periods of half-an-hour at a time, the fluid being removed by decantation, and the tissue well washed after each period of boiling. The extracts always yielded satisfactory jellies. After 3 hours the collagen had all been converted into gelatin, leaving only a powder which I found to be carbonate of lime with hardly a trace of organic matter; it gave the faintest possible xanthoproteic reaction.

Exp. II. In this instance the tissue was not subjected to the action of lime-water, nor to pancreatic extract. 25 grms. of finely divided tendon were boiled in 250 c.c. of water; the water was renewed once an hour or oftener for 7 hours, when the tissue was nearly all dissolved. The small residue examined microscopically proved to be of the structure of tendon fibres and showed the rows of shrunken tendon cells. The extract after the first 20 minutes of boiling, yielded, on concentration, a firm jelly, showing that some conversion of collagen to gelatin had already taken place during that short time, in spite of Siegfried's statement to the contrary.

It would seem from these experiments that one of the results of the action of pancreatic digestion is to render collagen more easily convertible into gelatin. Siegfried notices, in the case of the *mucosa* of the dog, that previous treatment with pancreatic extract causes reticular tissue to fall to pieces on boiling much more rapidly than undigested tissue, and he says he has performed other experiments to show that pancreatic digestion has an influence on the rapidity of formation of gelatin from reticular tissue.

Failing to detect reticulin in tendon, I endeavoured to obtain it from reticular tissue itself. I procured the intestines of two pigs, but in each case I found that the *mucosa* was difficult to distinguish and to separate from the *submucosa*, and that it was very thin, and I have, therefore, preferred to work with the tissues of dog and cat. In these animals

¹ I am indebted to Mr Benger for specially active preparations of *liquor pancreaticus*.

² The acetic acid mentioned above was never in sufficient quantity to render the tendon acid to litmus.

the *mucosa* is thick and very easily distinguished and separated from the whiter and firmer *submucosa*. As digestive fluids I have used either Benger's *liquor pancreaticus* (usually in 25 % solution in 0.75 % sodium carbonate), or else Parke Davis and Co.'s pancreatin which I obtained direct from the manufacturers at Detroit; of this I used generally 2.5 grs. to a litre of 0.5 % sodium bicarbonate with some carbonate, this being a much larger proportion of pancreatin than Siegfried used. The mucous membrane contains a considerable quantity of mucin, which cannot be removed by simply washing for a few hours. This forms a slimy mass when alkali is added and impedes digestion. I therefore removed the greater part of the mucin before digestion by soaking the tissue for 24 hours in a 1 % solution of sodium carbonate, and then washing, repeating the treatment for a second period of 24 hours in some cases. It is remarkable that Siegfried makes no mention of the presence of mucin or mucin-like substances.

I have usually found that digestion of the cell substance takes much longer than the time Siegfried allowed, often as much as a fortnight, the digestive fluid being renewed every few days; and in many cases I have regarded it as impossible to remove the remains of broken-down cells; and this especially so, when I have attempted further digestion after the tissue has been extracted with ether.

When digested tissue is boiled 24 hours with water I have always obtained a minute residue which gives the xanthoproteic reaction, and is probably coagulated undigested proteid or some substance corresponding to anti-albumid.

The following are typical of many experiments performed:

EXP. III. The mucous membrane of the small intestine of a cat was washed in water and put into 250 c.c. of 1 % sodium carbonate over night; it was next day washed until no longer slimy, and put into 400 c.c. water in which 2 grs. sodium bicarbonate, 1 gr. carbonate, and 1 gr. Parke Davis and Co.'s pancreatin were dissolved, chloroform being used as antiseptic. The whole was kept at 37° C. for 5 days. The tissue was washed, and boiled in 50 c.c. water for 20 minutes; the extract was removed by decantation and was concentrated. The tissue was washed and then boiled with 50 c.c. fresh water; and so on repeatedly. Each extract before concentration gave a precipitate with tannin; the first 6 extracts mixed together yielded a jelly; the 7th, 8th, and 9th extracts together yielded a jelly; the 10th gave a small quantity of tender jelly, and so did the 12th extract. After boiling the tissue for 2 hours, 40 minutes the fluid became more difficult to decant, as the

pieces of tissue were softer, but they were still curled as when the boiling commenced. The tissue now appeared to gradually wear away, little pieces breaking off; but there was never at any time a falling to powder as described by Siegfried; and the fact of obtaining a jelly from the 12th extract shows that all the gelatin-yielding substance had not been converted into gelatin in 3 hours, 40 minutes. After boiling all night (with a long vertical tube fitted into the cork of the flask to avoid loss by evaporation), a small residue was left, which gave the xanthoproteic reaction. This was not reticulin, for according to Siegfried, reticulin when boiled for many hours continuously forms an opalescent solution.

The next experiment is one in which the mucous membrane was extracted with ether.

EXP. IV. The mucous membrane of the small intestine of a dog was soaked for 24 hours in 500 c.c. of 1% sodium carbonate solution, and was then digested, after washing, with 400 c.c. of 0.25% pancreatin in 0.5% sodium bicarbonate solution for 4 days and again for further periods of 7 and 4 days. The tissue having been digested for 15 days, was well washed with water and then with alcohol, and was left during 23 days in ether. After this treatment the tissue was boiled during about 8 hours in successive volumes of water. The amount of water used during each operation was 50 c.c., and the water was changed at intervals of from 20 to 80 minutes. After such boiling, the tissue was still unchanged in appearance, and the extracts did not yield jellies, even when several were concentrated together. The tissue was allowed to boil all night in 100 c.c. water and there was next day a slight residue; the fluid in which it had been boiled gave a precipitate with acetic and with nitric acid, and with tannin, and gave a distinct xanthoproteic reaction.

From this experiment it would seem that prolonged treatment with ether had hindered the formation of gelatin when boiling water acted on the tissue; and I proceeded to test this quantitatively.

EXP. V. The mucous membrane of the small intestine of a cat was soaked for a few hours in 1% solution of sodium carbonate and was afterwards well washed in water and each piece was wiped with linen. It was divided into two equal parts (12.9 grs. in each). One part was put into 400 c.c. of water already boiling and kept at 98° C. for 4 hours. Small pieces of tissue (probably villi) became detached as soon as the tissue was put into boiling water. At the end of the 4 hours these were collected on a dried weighed filter, and they were later washed with water till there was no longer any cloudiness when tannin was added to the washings; afterwards with alcohol and with ether; the filter was then dried at 110° C. and

weighed. The tissue weighed 0.02 gr. The larger pieces of tissue were severally washed well in water and were left in strong alcohol for 18 hours, in absolute alcohol for one hour and in ether for 7 days; they were then dried at 110° C. and weighed. The weight was 0.35 gr. Adding to this the weight of the small pieces, the whole weight of residue was 0.37 gr.

The other portion of mucous membrane was put into strong alcohol for 1 hour, absolute alcohol for 1.5 hours and ether for 7 days. It was then heated in 400 c.c. water at 98° C. for 4 hours—no small pieces broke off as in the case of tissue not previously hardened with ether—the residue was washed with water and left in strong alcohol 18 hours, absolute alcohol 1 hour, and ether half-an-hour, after which it was dried at 100° C. and weighed. The weight was 0.45 gr.

It will be observed that the tissue was in each case under ether for the same length of time, so the loss of weight due to the removal of fat was in each case the same. The difference in weight may at first sight appear small; but it must be remembered that only a portion of the mucous membrane is composed of a gelatin-yielding material, there being much proteid present.

It now seemed desirable to ascertain whether ether has a similar "coagulating" effect on tendon fibres. Prof. Halliburton tells me that some years ago Dr T. G. Brodie performed in this laboratory some experiments with alcohol. The main results of Dr Brodie's work, hitherto unpublished, are as follows:

(1) Alcohol has practically no "coagulating" action on gelatin. Gelatin may be kept for weeks or months under alcohol, but after that lapse of time it is still readily soluble in hot water and the solution gelatinizes on cooling.

(2) The effect of alcohol on collagen is, however, different. Even after a few days under spirit it is difficult to extract much gelatin with boiling water. After a few months none at all can be obtained.

I have now found that ether has a similar effect on collagen. The following experiment shows the result in a quantitative way:

EXP. VI. Some tendon was finely divided and in the fresh condition three portions of 20.0 grs. each were weighed. One portion was put into alcohol direct. Another portion was stirred for a quarter of an hour in alcohol and was then put into ether. The third portion was treated as follows:

The tendon was put into a flask and 600 c.c. of water already boiling were added, and the whole kept at 98.5° C. for 3½ hours, the flask being fitted with a cork and a long upright tube to prevent loss by evaporation.

The contents of the flask were poured on to a filter, previously dried to constant weight at 110° C., and the residue, being collected on a filter, was washed with cold water until the washings no longer gave any cloudiness with tannin. It was then washed with spirit (the tube of the funnel being fitted with a cork) which was allowed to remain all night. Next day the spirit was allowed to drain through, and the residue on the filter was treated with absolute alcohol for an hour, and with ether for a quarter of an hour. It was very hard and appeared dry; it was now heated in an oven at 100° C. until the weight was constant.

As a result of heating 20.0 gr. of moist tendon with 600 c.c. water at 98.5° C. for 3½ hours, I obtained 4.00 grs. of dry residue.

(In the above experiment I always used cold water for washing the tendon; because hot water, even when it does not boil, converts collagen to gelatin, as I showed thus: 5 grms. of tendon were kept in 25 c.c. water in presence of chloroform at 60° C. for 23 hours. The clear liquid was separated and the albumin in it precipitated by boiling with a few drops of acetic acid. This precipitate was filtered off and tannin was added to a portion of the filtrate; a heavy precipitate was produced; the remainder of the filtrate on concentration yielded a firm jelly.)

The portion of tendon which was left under spirit was after 45 days dried at ordinary temperature and treated with water exactly as above. The residue weighed 6.11 grs.

The portion which was left under ether was after 43 days treated in exactly the same way; 6.54 grs. was left.

This experiment proves quantitatively that ether and alcohol, acting for a prolonged period, render collagen much less easily convertible into gelatin.

In no experiment on the boiling of reticular tissue with water, have I observed the falling to a powder described by Siegfried. On boiling the *mucosa* of dog after pancreatic digestion, he reports the falling to pieces in a quarter of an hour, whereas, if it were not previously digested this did not take place for one hour. I have also boiled undigested tissue, but even after 4 hours it was apparently unchanged; though when the tissue has been digested and not extracted with ether, I have noticed that it gets softer and begins to wear away during the second or third hour of boiling.

In no case have I extracted nearly all the gelatin that can be obtained from a sample in 20 minutes as described by Siegfried. Where I have obtained jelly from the first extract I have obtained it in apparently equal quantities from later extracts; and where the tissue has been for a prolonged period under ether I have not obtained more than traces of gelatin in any extract.

Siegfried says that he boiled reticulin for 72 hours with hydrochloric acid and tin chloride, and after removal of the tin saturated the concentrated fluid with hydrochloric acid at 0° C. After standing for many days in one instance no glutaminic hydrochloride had crystallized out, and in another instance there was only a trace.

Not being able to obtain reticulin, I have treated reticular tissue, after it has been boiled in water $1\frac{1}{2}$ —2 hours (which presumably Siegfried would consider long enough to remove all the gelatin-yielding substance), in a similar way, and have always been successful in obtaining a considerable deposit of small crystalline needles, which I believe to be glutaminic hydrochloride.

As one of many experiments I will cite the following:

Exp. VII. Some mucous membrane of the small intestine (cat) after digestion with 25% Benger's *liquor pancreaticus* in 0.75% sodium carbonate for nearly 3 weeks, the digestive fluid being renewed every few days, was washed in water and left to soak in distilled water, several times changed, in presence of chloroform, for 7 days. The prolonged washing with water was to remove the digestive fluid and products of digestion as far as possible. When examined microscopically the tissue appeared to be particularly free from cell substance. After treating for 2 hours with alcohol, the tissue was kept under ether for 7 days. It was dried at the temperature of the laboratory and its weight was 0.27 grms. This quantity was obtained from the mucous membrane of 9 cats.

The dried tissue was boiled in 50 c.c. of water for four periods of half-an-hour, being well washed after each period of boiling. From every extract I obtained a firm jelly. Since Siegfried states that almost all the gelatin is removed in 20 minutes, I conclude that if glutaminic hydrochloride could now be produced, he would not consider it to have come from any remaining gelatin-yielding substance.

After boiling for 72 hours with 50 c.c. of 15% hydrochloric acid and a little tin dichloride, subsequently removing the tin by sulphuretted hydrogen, and concentrating the fluid to about 20 c.c., I saturated with hydrochloric acid gas. In a few minutes there was a deposit of cubical crystals of an inorganic nature—presumably of sodium chloride—and later, after surrounding the vessel containing the fluid with a mixture of ice and salt, I obtained a considerable deposit of small needles.

It is not possible to state positively without an elementary analysis that these crystals consist of glutaminic hydrochloride. The small amount of material at my disposal rendered this impossible; the difficulty of obtaining a larger yield will be evident from the fact that

the mucous membrane of 9 cats only gave me 0.27 gr. of raw material to start with. I, however, consider it extremely probable that they did consist of glutaminic hydrochloride; and my evidence is that their method of formation at a low temperature and their ready solubility on exposure to air when the hydrochloric acid evaporates, seems to preclude the possibility that they can be anything else. In crystalline form also they are similar to those which can be obtained from gelatin. I prepared crystals from gelatin itself under the same conditions, and in all the foregoing points they were identical with those obtained from the reticular fibres. The crystals obtained from gelatin I purified by recrystallizing twice, and determined the percentage of nitrogen in them by Kjeldahl's method. The results of two experiments were 7.51 and 7.57 per cent. of nitrogen. The calculated percentage of nitrogen in glutaminic hydrochloride is 7.64, and I think I am justified in concluding that glutaminic hydrochloride is the body with which I have been dealing.

Horbaczewski¹ obtained glutaminic hydrochloride by the action of hydrochloric acid on gelatin, and this he identified by making carbon, hydrogen, and chlorine determinations.

CONCLUSIONS.

It will be seen that I have been able to confirm very few of Siegfried's statements; and my main result is that reticulin does not exist either in ordinary white fibrous tissue (tendon) or in the reticular tissue of the intestinal mucous membrane. Both consist of fibres which are chemically and histologically identical; the main material of which they are composed is the gelatin-yielding substance called collagen.

I regard Siegfried's reticulin merely as collagen which has been "coagulated" by the reagents he employed (especially alcohol and ether) *plus* proteid and nuclein residues of cells. After treatment with these reagents the conversion into gelatin is much more difficult, not only in the case of a finely stranded tissue like reticular tissue, but even in such a dense material as tendon.

The fact that Siegfried's reticulin contains a small amount of phosphorus I attribute to the nuclei and other residue of cells. These (especially the cells of Lieberkühn's crypts) are much more difficult to get rid of by pancreatic digestion than Siegfried considers; on

¹ *Akad. d. Wiss. Sitzber. Wien.* LXXX. 2, p. 117. 1879.

microscopical examination of the digested tissue many parts will be found free or all but free from cells, whereas on making further search other pieces of the same mucous membrane will show a great many; on the whole, this is more marked in the dog than in the cat. Siegfried himself states that the phosphorus cannot be contained as nuclein because shaking reticulín with dilute nitric acid at 25° C. for half-an-hour will not produce phosphoric acid. Prof. Halliburton's experience with nuclein from animal cells is that considerably more vigorous treatment than this is necessary to produce phosphoric acid from it.

Siegfried states that reticulín yields to dilute alkali an organic compound containing phosphorus, soluble in chloroform and alcohol, and this he is inclined to attribute to something that resembles lecithin; the body in question might on my hypothesis be also derived from cell-residues.

With regard to the high percentage of sulphur, I would suggest that as he had previously removed from the tissue part of the collagen as gelatin (a substance poor in sulphur) the residue which he termed reticulín would contain a relatively high proportion of sulphur on account of the proteid admixture which would not have been removed by the boiling.

Coming next to the decomposition products of Siegfried's reticulín, he states that glutaminic acid is absent. I have certainly always found a body which I believe to be glutaminic hydrochloride in the decomposition products of reticular tissue which has been treated in a manner which Siegfried states to be sufficient to remove all collagen.

I have already alluded to the difficulty of being quite sure on this point, for glutaminic acid gives no tests by which it may be readily recognized, and the amount I obtained was too small for elementary analysis.

There are some small points on which my work differs from that of Siegfried; for instance, as to the relative length of time necessary to obtain gelatin from white fibrous tissue and reticular tissue respectively. I have also never observed the falling to pieces of reticular tissue which Siegfried states occurs on boiling it.