HISTOCHEMICAL STUDIES ON THE NATURE AND FORMATION OF EGG CAPSULES OF THE SHARK CHILOSCYLLIUM GRISEUM [1]

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Pryor (1940) demonstrated that the ootheca of *Blatta* is composed of a tanned protein similar to that of insect cuticle. Since then a number of authors reported the occurrence of tanned proteins in the cysts of nematodes, egg shells of helminths, setae of earthworm and byssus of *Mytilus edulis* (Ellenby, 1946; Stephenson, 1947; Dennell, 1949; Brown, 1952; Smyth, 1954). In this connection it is of interest to recall the observation of Brown (1950a) that the egg capsules of selachians also give evidence of phenolic tanning although it was believed by the earlier workers to be formed of a material similar to keratin. In the light of these observations it was thought that a study of the nature and composition of the egg capsules of shark would be of interest in itself and for comparison with the egg shells of helminths and ootheca of *Blatta* which have been shown to possess a protein constitution resembling in essential respects that of tanned cuticles of insects.

The materials used in the following study comprise nidamental glands and egg capsules collected from gravid females of *Chiloscyllium griseum*. The staining and histochemical reagents used are mentioned in relevant context. For localization of the oxidase of the egg capsule, the "catechol" technique (Smyth, 1954) was applied. The microchemical and chromatographic procedures employed for the study of the protein of the egg capsule are described in the text.

Egg Capsule—Structure

The egg capsules of sharks and rays have been described by a number of workers (Beard, 1890; Widakowich, 1906; Clark, 1922; Hobson, 1930; Nalini, 1940). The shell material is said to be secreted by the cells of the nidamental gland and the egg capsule is formed in the caudal part of the oviduct. The sequence of events in the formation of the egg case is not known for certain. There is some evidence to suggest that a major part of the egg case is formed before the arrival of the fertilized egg. The egg case with the enclosed egg is later ejected into the sea.

The egg capsules of sharks are more or less rectangular in shape with the corners prolonged into anterior and posterior pairs of horns, but considerable variation exists in shape and size in the different species. There is little precise information regarding the nature of the material composing the egg capsule. The earlier workers referred to it as chitinous but the term as used by them carried no chemical significance. On the other hand Hussakof and Welker (1908) who re-

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ported on the chemical nature of the egg case of two species of sharks, suggested that it may be similar to keratin. However, the previous workers are agreed that in all selachians the structure and composition of the egg capsule are identical. Widakowich (1906) noted that in *Scyllium* the egg capsule is formed of a large number of "Platten" which adhere to each other loosely at first and later, especially after contact with sea water, much more closely so that the entire capsule hardens. It has also been observed that the capsule when first formed within the oviduct is white and soft and gradually hardens, undergoing a change in color to brown and later to deep reddish brown. In *Chiloscyllium griseum* the egg capsules taken from the oviducts show a range of coloration varying from very light brown to deep reddish brown. The ridge-like thickening bordering the capsule is more deeply colored than the rest of it.

Frozen sections of the capsule wall, which is lightly colored, show an outermost narrow yellowish layer containing dark granular inclusions. Internal to it is a

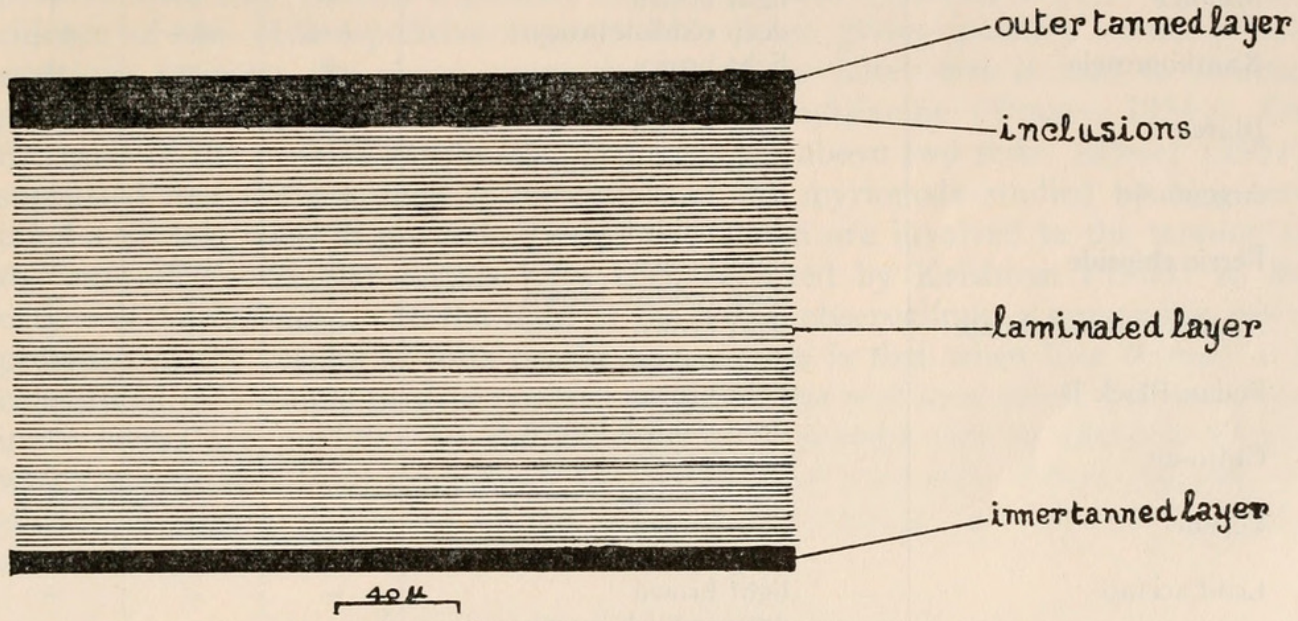

FIGURE 1. Section through the wall of a light colored egg capsule stained with Mallory.

broad region more or less uncolored and characterized by horizontal laminations. On the inner border of the laminated region is a very narrow strip which is yellow colored and apparently homogeneous, similar to the outermost layer (Fig. 1). In the laminated region a central part may be distinguished by its darker shade. The color disappears after treatment with ethylene chlorhydrin and the entire section is marked by a diminution of the dark color. This reaction may suggest the presence of melanin-like substances in the wall of the capsule (Lea, 1945). Further support to the above suggestion is obtained by the results of tests performed on the sections of the capsule wall with hydrogen peroxide and potassium permanganate, both of which produce a bleaching effect (Pearse, 1954).

In paraffin sections stained with Mallory, the outer- and innermost layers, which are yellowish in unstained preparations, are colored red with acid fuchsin while the laminated region is stained blue. Similar results were obtained with Masson's trichrome stain; the regions coloring red with Mallory are stained with xylidene ponceau and the laminated region is colored green. With Heidenhain's haema-

toxylin the outer and inner layers are dark blue while the rest of the thickness of the wall comprising the laminated zone is very lightly or not at all stained. In the regions of the capsule which are reddish brown, the staining reactions are different from those reported above. The central wide laminated region, instead of staining uniformly green or blue with Masson's and Mallory stains, shows red patches filling up the greater extent of this region. Such a change in staining reaction may suggest that the substance originally present has undergone a transformation so as to resemble that present in the outermost layer.

TABLE I

Responses of egg capsule wall of Chiloscyllium griseum to chemical tests

Test	Egg capsule wall	Outer layer	Middle laminated zone	Inner layer
Millon's	light brown	+	−	+
	deep reddish brown	++	++	++
Xanthoproteic	light brown	+	−	+
	deep reddish brown	++	++	++
Biuret	light brown	−	+	−
	deep reddish brown	−	−	−
Argentaffin	light brown	++	+	++
	deep reddish brown	++	++	++
Ferric chloride	light brown	+	++	+
	deep reddish brown	+	+	−
Ammonium molybdate	light brown	+	+	+
	deep reddish brown	+	+	+
Sudan Black B	light brown	−	−	−
	deep reddish brown	−	−	−
Chitosan	light brown	−	−	−
	deep reddish brown	−	−	−
Pepsin	light brown	−	+	−
	deep reddish brown	−	−	−
Lead acetate	light brown	−	−	−
	deep reddish brown	−	−	−
Sodium nitroprusside	light brown	−	−	−
	deep reddish brown	−	−	−
Dilute mineral acids	light brown	−	+	−
	deep reddish brown	−	−	−
Boiling H_2O	light brown	−	+	−
	deep reddish brown	−	−	−

+ = positive.
− = no apparent effect.

CHEMICAL COMPOSITION

To test the validity of the above assumption, the nature of the principal chemical components of the capsule walls yielding the staining reaction reported above was investigated. It is known from previous work that the major constituent of the shell substance is protein. In the following study a qualitative estimate of the protein constituents was made by color tests and the results are summarized in Table I. It is seen that the entire thickness of the capsule wall is positive to tests for protein. But the light colored capsules differ in some respects from those

which are deep reddish brown. It has been pointed out that the capsule when first formed is white and later turns to light brown which gradually deepens to dark reddish brown, the changes in coloration representing different phases in the growth of the capsule. In the earlier phases of growth when the capsule is very light brown, the outer and innermost layers give positive Millon's and xanthoproteic reactions, while the central laminated region is negative to these tests, but positive to biuret test. In the more fully formed capsule, which is deep reddish brown, the central laminated zone also is positive to Millon's and xanthoproteic tests. These changes in the reaction to protein color tests coincide with those in staining reactions with Mallory. Presumably the fuchsinophil substance staining red with Mallory is the same as that giving a Millon-positive reaction.

A positive reaction to Millon's test has been interpreted as indicative of a protein containing the hydroxyl-phenyl group in the molecule, and since tyrosine is the only amino acid containing it, it may be inferred that in the egg capsule Millon-positive sites may contain a protein rich in tyrosine (Pearse, 1954). The coincidence of the Millon-positive regions with those giving positive xanthoproteic reactions supports the above suggestion since the latter test is said to indicate protein containing tyrosine, tryptophane and phenylalanine (Pearse, 1954). On the basis of the positive results obtained with the above two tests, Blower (1951) suggested that the presumptive exocuticle of the myriopods studied by him contains a protein, rich in phenolic groups and which are involved in the tanning of the exocuticle. Similar results have been reported by Krishnan (1956) in the cuticle of *Scolopendra*. In the light of the above observations, a reasonable interpretation of the results of tests on the egg capsule is that when first formed it is constituted of a simple protein positive to biuret test and soon after, the outer and inner layers are modified by the presence of a protein rich in phenolic groups which appear to spread throughout the thickness of the wall. These changes are correlated with a deepening of the color of the capsule and also an increased chemical resistance.

Evidence for Quinone-Tanning

The features noted above recall strongly the characteristic change undergone by the insect cuticle during hardening by tanning (Dennell and Malek, 1955) and appear significant as indicative of a similar tanning process in the egg capsules. Brown (1950b) pointed out that if a structural protein dissolves only in sodium hypochlorite solution and is itself secreted by tissues containing polyphenols, there is circumstantial evidence for quinone tanning. It will be shown in the sequel that both the tests are positive with the egg case material. When small pieces of dark brown capsule wall were treated for varying periods with a dilute aqueous solution of sodium hypochlorite, the color was readily lost and on continued treatment the egg capsule wall was dissolved. Further evidence of tanning is indicated by the presence of a phenol oxidase which is known to be an essential participant of the tanning process in insect and crustacean cuticles (Bagvat and Richter, 1938; Dennell, 1947a, 1947b; Krishnan, 1951). In recent studies on the formation of helminth egg shell, which involves quinone tanning, the oxidase concerned has been demonstrated by the red color produced on incubation with a dilute solution of catechol (Smyth, 1954). The mechanism of the above reaction is due to the

oxidation of catechol to quinone and its condensation with the protein so as to produce a tanning effect. This technique was applied to the egg capsules of shark with positive results. Light colored capsule walls when subjected to the catechol treatment changed to a dark red color resulting from the tanning of the protein. Such a color change is less intense with dark colored capsules. That the change in color after catechol treatment is really due to the tanning of the protein may be inferred from the observation that the color is lost on addition of a dilute solution of sodium hypochlorite. Further, sections of material deeply colored by catechol treatment when stained with Mallory show correlated change in staining reaction, the central laminated region being fuchsinophil, so as to simulate the condition of a more full grown and normally reddish brown capsule. It would appear that by treatment with catechol the protein of the central layer is artificially tanned. The color change noted above was inhibited by cyanide in a concentration of 0.001 *M*, suggesting the enzymatic nature of the process and the role of an oxidase in bringing about the tanning effect.

The above observations suggest that the egg capsule after its formation undergoes a process of hardening by phenolic tanning before being ejected into the sea, and this would account for the change of its coloration from light brown to deep reddish brown. The principal participants in the process appear to be a protein probably rich in tyrosine and a phenol oxidase. The results of histochemical tests on the egg capsule material (Table I) indicate an absence of lipids which are usually associated with tanning in the cuticles of insects and other arthropods. Further, although diphenols are indicated in the capsule walls, as may be inferred from positive ferric chloride tests, in the absence of a correlation between their accumulation and the tanning of the protein, their mere presence may not indicate that they are involved in tanning. Their persistence in the outermost layer, where tanning is more intense than in the rest of the thickness of the wall, may militate against the view that they are the tanning phenols. It is suggested that they may be related to the formation of melanin occurring in the capsule walls, for diphenols, indicated by ferric chloride, accumulate in the laminated zone early in the growth of the capsule and their partial disappearance is followed by the occurrence of melanin. This feature, together with the presence of a phenol oxidase in the capsule wall, may suggest the oxidation of phenols to melanin. Since the latter appears even before the onset of tanning in this region it is suggested that the free diphenols may not be directly involved in the tanning process.

Histochemistry of the Nidamental Gland

With a view to investigate further the nature of the tanning process, a study of the mode of formation of the egg shell material was made. It is known from previous work that the materials forming the egg case are secreted by the nidamental gland. The gland is a dilatation of the oviduct at the junction of the caudal and cranial parts comprising a glandular body formed of tubules in a more or less parallel series. The histology of the gland in *Chiloscyllium* shows close agreement with that of *Scyllium canicula* and *Scyllium catalus* (Nalini, 1940). In the anterior part of the gland, distinguished as the albumin gland, the secretory tubules are formed of both glandular and ciliated cells. The secretions are in the form of transparent cytoplasmic granules which appear to be extruded by rupture of the cell

walls into the lumen of the gland tube. The succeeding section of the gland, which is distinguished as the shell-secreting zone, is formed of cells whose cytoplasm is packed with granules during the period when egg capsules are being formed. The shell substance appears to be derived from these secretions and in the light of the foregoing observations on the egg capsules, one would expect to find in these cells the constituents of the tanning system. Accordingly histochemical tests for phenols and proteins were applied. The argentaffin, ammonium molybdate and sodium iodate tests for phenols were positive in the cytoplasm of the cells of the shell-secreting zone. Identical regions of the cells were also positive to biuret tests for protein. Malachite green, which is known to show a specificity for proteins involved in tanning of egg shells of helminths (Johri and Smyth, 1956), gave positive reaction in the cytoplasm, the granules taking a vivid green color. The green coloration is said to be due to the dye becoming bonded to the protein. Since the cytoplasmic granules in the cells of this region react positively to both the tests for phenols and proteins, it is suggestive that the substance reacting may be a phenolic protein, similar to that reported to occur in the vitelline gland cells of helminths (Smyth, 1954). Frozen sections of this region of the nidamental gland when treated with a dilute solution of catechol develop readily a brown coloration in the cytoplasm of the cells. This reaction may suggest evidence of the occurrence of a protein undergoing tanning and an oxidase in close proximity to it, responsible for the oxidation of phenols involved in tanning. It appears probable that the oxidase and the substrate are both located in the cytoplasm of these cells.

Nature of Egg Capsule Protein

The foregoing observations indicate that the principal constituent of the egg capsule is a protein secreted by the cells of the nidamental gland, along with an oxidase capable of oxidizing catechol to quinone. In the egg capsules two apparently distinct protein constituents seem to occur, one forming the basal matrix which persists for some time in the central laminated region and the other being a tanned protein which is distinguished from the former by the chemical and staining reactions. In these respects they present very strong resemblance to the basal protein and that impregnating the regions destined to be tanned in the cuticle of insects like *Periplaneta* (Dennell and Malek, 1955). Here the basal protein of the procuticle stains blue with Mallory, is negative to Millon and xanthoproteic tests and lacks chemical resistance, while that impregnating the presumptive exocuticle stains red with Mallory, is positive to Millon and xanthoproteic tests and possesses considerable chemical stability. That the above characteristics of the protein of the presumptive exocuticle may be due to some sort of aromatic bonding is suggested by the observation of Kennaugh (see Dennell, 1958) that the staining properties can be reversed by treatment with Diaphanol which is known to break up the aromatic bonds by oxidation. The change in staining reaction with Mallory from red to blue, reported by the above author, may indicate a restoration of the protein component to its original state, as is found in the untanned endocuticle. In the egg capsule of the shark it is suggestive that the tanned protein is derived from the basal protein such as is found in the laminated region in the earlier stages of capsule formation. If it is so, it may be possible to restore the tanned protein to the original state by breaking up the aromatic bonds as has been done in the insect

cuticle referred to above. This was carried out by adopting the method used by Dennell (1958) who following Trim (1941), separated the tanning phenols of the puparia of *Calliphora* using alkaline stannite solution for breaking up the quinone bonds. Accordingly, small pieces of egg capsule material were left in a mixture of 2% sodium hydroxide and stannous chloride at 37° C. for nearly a week, by which time the protein was solubilized. The protein fraction was separated and tested. Unlike the tanned protein it was negative to Millon's test and was easily digested by pepsin-hydrochloric acid and showed a marked swelling in boiling water.

These observations, in addition to suggesting that the tanned protein of the egg capsule may be a derivative of the basal protein, also give some indication of the nature of the protein. The reaction to pepsin and swelling in boiling water are suggestive, especially in the light of the observation of Astbury (1945) that the entire egg capsule of shark yields an x-ray diffraction pattern similar to that of a collagenous protein.

With a view to test further the suggestion made above, a microchemical analysis of the capsule protein was made using a modification of the method of Spencer, Morgulis and Wilder (1937), who applied the above method for a determination of collagen content of the muscles of rabbit. The capsule walls were cut into small bits and cleaned by scraping with a blunt scalpel to remove all adhering tissue. A sample weighing 0.1 gm. was homogenized with an equal quantity of distilled water in a Potter-Elvehjm homogenizer, and the material was placed in a water bath at 100° C. for about 15 minutes along with 10 times its weight of water. This was later stored in a refrigerator, and next day, it was autoclaved for 3 hours at 20 pounds pressure so as to convert collagen, if any, into gelatin. The material was then centrifuged at 4,000 rev./min. for one hour and the supernatant fluid drawn off. An aliquot of this fluid was treated with 3% tannic acid when a copious precipitate was obtained. The above evidence in support of the view that a collagenous type of protein occurs in the egg capsule was checked by a chromatographic analysis of the precipitate. The material was treated with ten times its weight of 6 N HCl in a sealed tube and hydrolysed at 105° C. for 24 hours. The hydrolysate was dried in a vacuum desiccator containing potassium hydroxide and this was used for analysis by partition chromatography, following the capillary ascent method of Williams and Kirby (1948). The hydrolysate was dissolved in a small quantity of distilled water and a 20-μl sample was used for spotting on the filter paper and the chromatogram run with butanol-acetic acid-water as the solvent. Simultaneously a number of chromatograms were run under identical conditions using pure amino acids for purposes of comparison. A solution of 0.1% ninhydrin in butanol was used for spraying. Qualitative analysis of the chromatogram thus obtained shows in general an agreement in amino acid make-up with that of mammalian connective tissue (Bowes and Kenten, 1949), suggesting that the protein in question may be allied to collagen. Further, the pattern of spots was more or less identical with that of a sample of pure gelatin hydrolysed and otherwise treated in the same way as the test material. Most of the amino acids found in the chromatogram of the egg case material correspond to those found in the gelatin.

However, it is seen that the egg case material differs in the absence of hydroxylysine, leucine and valine as well as in the presence of tryptophane. The absence of hydroxylysine may suggest a relationship to elastin, but the occurrence of tryptophane is unusual for a collagenous type of protein. It is possible that its

presence may be due to a contaminant. However, it must be mentioned that the amino acid composition of collagen derived from different sources may vary markedly. The collagen of fish skin is known to differ from mammalian collagen in having a low hydroxyproline content while serine, threonine and methionine are in greater amounts (Gustavson, 1956). Such quantitative variations occur not only in those amino acids considered to be characteristic of collagen but also in some of the non-typical residues like tyrosine. A quantitative amino acid analysis is therefore necessary for making a valid comparison. However, the present study is essentially from a biological viewpoint and such evidence as has been obtained is enough to indicate the nature of the material composing the egg capsule. The presence of non-polar amino acids like glycine and alanine, the prominence of proline and hydroxyproline and the comparative rarity of aromatic residues are features of the egg capsule protein, which together are suggestive that it may be allied to the collagen group (Gustavson, 1956).

Discussion

The foregoing observations suggest that the egg capsules of *Chiloscyllium* undergo a tanning process resulting in acquisition of mechanical rigidity and chemical resistance. The process is comparable to that occurring during the formation of ootheca of *Blatta* (Pryor, 1940) but certain differences are significant. Unlike in the insect, here the substrate involved in tanning is a protein without a lipid component. No free diphenol appears to participate in the process. The resultant tanned product is also different from the tough amber colored sclerotin, being only yellowish, and retains a reactivity to stains. The protein constitution of the egg capsule appears to be such that it cannot yield sclerotin after tanning, for it has been observed in arthropod cuticles that unless the protein precursor of tanning is impregnated with a lipid constituent, sclerotin may not be the resultant product. Sclerotin itself has been considered as a lipoprotein subsequently tanned. It is clear that the tanned protein of the egg case is not sclerotin but recalls in its chemical and staining reactions the tyrosine-rich protein precursor of sclerotin, found in the presumptive exocuticle of an inset like *Periplaneta* (Dennell and Malek, 1955) or the so-called pro-sclerotin described by Blower (1951) in the myriopods studied by him. In the above instances the protein in question shows considerable chemical stability even before forming a complex with the lipid participant of tanning and stains red with Mallory, unlike the protein confined to those regions which do not undergo tanning. The chemical stability of the protein has been attributed to the occurrence even at this stage of some kind of aromatic tanning which is distinct from the subsequent tanning of the lipoprotein complex by free diphenols resulting in sclerotin (Dennell, 1958). Such a tanning has been distinguished by the above author as "primary tanning" in contrast to the "secondary tanning" which results in sclerotin. In the absence of the participation of free diphenols "primary tanning" would be presumably by oxidation of tyrosine side-chains of the protein. The tanned protein of the shark egg capsule recalls strongly the product of "primary tanning" in its chemical nature, staining characteristics, possession of resistant qualities and retention of a "tannable" condition having still free amino groups. It seems probable from the observations reported in the present study that the mode of tanning of the egg capsule protein may involve a process of auto-quinone tanning similar to that suggested to occur in the egg shells of helminths (Smyth, 1954).

Summary

1. The egg capsules of *Chiloscyllium griseum,* when first formed in the oviducts, are soft and white and gradually turn light brown to deep reddish brown before being ejected into the sea.

2. Light brown capsule walls show in section an outer and an inner narrow layer apparently homogeneous and yellowish in color while a wide central region is laminated and uncolored. This layer stains blue with Mallory, indicates the presence of a simple protein positive to biuret test and lacks chemical resistance. The outer and inner layers stain red with Mallory and contain a protein which is positive to Millon and xanthoproteic tests indicative of phenolic groups. In deeply colored walls the central laminated layer shows staining and histochemical reactions similar to those of the outer layer.

3. Evidence has been presented indicating that the above changes may be due to the tanning of a basal protein involving a phenol oxidase resident in the capsule wall.

4. The constituents of the tanning system are derived from the secretions of the cells of the nidamental gland. The tanning of the egg capsule protein does not appear to involve free diphenols so that some form of auto-quinone tanning seems to occur.

5. The tanned protein of the egg capsule is unlike the sclerotin of the insect cuticle, but recalls in its staining and histochemical reactions the protein precursor of tanning impregnating the presumptive exocuticle of insects like *Periplaneta.*

6. The nature of the egg capsule protein has been investigated using microchemical and chromatographic methods. From the results obtained it is suggested that it is allied to the collagen group of proteins.

7. The results are discussed.

LITERATURE CITED

Astbury, W. T., 1945. The forms of biological molecules. *In:* Essays on Growth and Form. Clarendon Press, Oxford.

Bagvat, K., and D. Richter, 1938. Animal phenolases and adrenalin. *Biochem. J.,* **32**: 1397–1400.

Beard, J., 1890. On the development of the common skate *Raia batis.* 8th Annual Report, Fisheries, Scotland.

Blower, G., 1951. A comparative study of the chilopod and diplopod cuticle. *Quart. J. Micr. Sci.,* **92**: 141–161.

Bowes, J. H., and R. H. Kenten, 1949. Some observations on the amino acid distribution of collagen, elastin and reticular tissue from different sources. *Biochem. J.,* **45**: 281–285.

Brown, C. H., 1950a. Quinone tanning in the animal kingdom. *Nature,* **165**: 275.

Brown, C. H., 1950b. A review of the methods available for the determination of the types of forces stabilizing structural proteins in animals. *Quart. J. Micr. Sci.,* **91**: 331–339.

Brown, C. H., 1952. Some structural proteins of *Mytilus edulis. Quart. J. Micr. Sci.,* **93**: 487–502.

Clark, R. S., 1922. Rays and skates—egg capsules and young. *J. Mar. Biol. Assoc.,* **12**: 577–543.

Dennell, R., 1947a. A study of an insect cuticle: the formation of puparium of *Sarcophaga falculata. Proc. Roy. Soc., London, Ser. B,* **134**: 79–110.

Dennell, R., 1947b. The occurrence and significance of phenolic hardening in the newly formed cuticle of Crustacea Decapoda. *Proc. Roy. Soc., London, Ser. B,* **134**: 485–503.

Dennell, R., 1949. Earthworm chaetae. *Nature,* **164**: 370.

Dennell, R., 1958. The hardening of insect cuticles. *Biol. Rev.,* **33**: 178–196.

DENNELL, R., AND S. R. A. MALEK, 1955. The cuticle of cockroach *Periplaneta americana*. *Proc. Roy. Soc., London, Ser. B,* **143**: 239–257; 414–434.

ELLENBY, C., 1946. Nature of the cyst wall of the potato-root eel worm *Heterodera rostochiensis* and its permeability to water. *Nature,* **157**: 302–303.

GUSTAVSON, K. H., 1956. The Chemistry and Reactivity of Collagen. Academic Press Inc., New York.

HOBSON, A. D., 1930. A note on the formation of the egg case of skate. *J. Mar. Biol. Assoc.,* **16**: 577–581.

HUSSAKOF, L., AND W. H. WELKER, 1908. Notes on the chemical nature of egg cases of two species of sharks. *J. Biol. Chem.,* **4**: XLIV–XLV.

JOHRI, L. N., AND J. D. SMYTH, 1956. A histochemical approach to the study of helminth morphology. *Parasitology,* **46**: 107–116.

KRISHNAN, G., 1951. Phenolic tanning and pigmentation of the cuticle in *Carcinus maenas*. *Quart. J. Micr. Sci.,* **92**: 333–342.

KRISHNAN, G., 1956. Nature and composition of the epicuticle of some arthropods. *Physiol. Zoöl.,* **29**: 324–335.

LEA, A. J., 1945. A neutral solvent for melanin. *Nature,* **156**: 478.

NALINI, K. P., 1940. Structure and function of the nidamental gland of *Chiloscyllium griseum*. *Proc. Ind. Acad. Sci.,* **12**: 189–214.

PEARSE, A. G. E., 1954. Histochemistry. J & A Churchill Ltd., London.

PRYOR, M. G. M., 1940. On the hardening of the ootheca of *Blatta orientalis*. *Proc. Roy. Soc., London, Ser. B,* **128**: 393–407.

SMYTH, J. D., 1954. A technique for the histochemical demonstration of polyphenol oxidase and its application to egg shell formation in helminths and byssus formation in *Mytilus*. *Quart. J. Micr. Sci.,* **95**: 139–152.

SPENCER, H. C., S. MORGULIS AND V. M. WILDER, 1937. A micro method for the determination of gelatin and a study of the collagen content of muscles from normal and dystrophic rabbits. *J. Biol. Chem.,* **120**: 257–266.

STEPHENSON, W., 1947. Physiological and histochemical observations on the adult liver fluke, *Fasciola hepatica*. *Parasitology,* **38**: 128–139.

TRIM, A. R. H., 1941. Studies on the chemistry of insect cuticle. *Biochem. J.,* **35**: 1088–1098.

WIDAKOWICH, V., 1906. Über Bau and Funktion des nidamental Organs von *Scyllium canicula*. *Zeitschr. f. wiss. Zool.,* **80**: 1–21.

WILLIAMS, R. J., AND H. KIRBY, 1948. Paper chromatography using capillary ascent. *Science,* **107**: 481–483.