The Formation of Tracheae and Tracheoles in *Rhodnius prolixus*

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With one plate (fig. 1)

SUMMARY

The formation of tracheae in *Rhodnius* is described by the hypothesis of expansion and buckling. The cuticulin lining is at first a smooth-walled cylinder. Later it expands equally in each direction, increasing in diameter but buckling in the axis. An engineering expression describing symmetrical buckling in a cylinder under uniform axial compression has been applied to this process. Agreement was obtained between the expected and observed values for buckling frequency and tube-wall thickness. The taenidia are formed within the buckles, their amplitude being proportional to the increase in diameter. The axial orientation of the chitin micelles in the lining membrane and the tangential orientation in the taenidia are consistent with their being oriented by the stresses expected during expansion and buckling. The formation of tracheoles may also be described by the expansion and buckling hypothesis.

INTRODUCTION

THE formation of tracheae has been discussed by Wigglesworth, (1931), Richards and Anderson (1942), Keister (1948), Richards and Korda (1950), and Richards (1951).

The characteristic pattern in the cuticle of tracheae and the origin of the taenidia have attracted the attention of numerous writers from the earliest microscopists onwards. Thompson (1929) was the first worker whose approach agreed with modern ideas. He said: 'Since the tracheal filament is continuous, the natural supposition is that it results from the operation of some simple physical laws and is produced by forces which are unaffected by the existence of cell boundaries in the tracheal epithelium, and act simply in the chitinous lining at the moment when it is being secreted.' Keister (1948) studied the development of tracheae in living *Sciara* larvae with a phase-contrast microscope but did not arrive at any hypothesis to account for their formation. The events she described are exactly those expected from the hypothesis of tracheal formation put forward in this paper. Wigglesworth (1954) has described moulting in *Rhodnius* tracheae. He compared the folding of the lining with the expansion of the epicuticle over the rest of the body, saying that the taenidia are a secondary secretion within the folds. Richards had previously rejected this hypothesis because of his belief in the presence of a continuous basal procuticle. No such layer is present in *Rhodnius* and the hypothesis of expansion is here elaborated. In *Rhodnius* the tracheae consist of a two-layered lining membrane folded round three sides of the taenidia, the annular or

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helical thickenings which separate the membrane from the epithelium (fig. 1, c). Both the outer layer of the membrane and the taenidia contain chitin, in the former with the micelles axially oriented and in the latter with the micelles lying tangentially (Locke, 1957). The problem is to account for (1) the regular helical and annular folding of the cuticle, (2) the formation of the taenidia and their micelle orientation, and (3) the axial orientation of micelles in the lining membrane.

The advent of the electron microscope has shown that tracheoles have a structure similar to that of tracheae—a lining membrane supported by annular or helical thickenings, but too small for the micelle orientations to be determined. This obvious similarity between tracheae and tracheoles suggested that the same mechanism might be operating in their growth.

Material and Methods

At 25° C 4th instar larvae of *Rhodnius prolixus* Ståhl moult 14–15 days after feeding. A series showing stages in the formation of a trachea was obtained by fixing at intervals. The usual procedure was to dissect under Ringer and to fix and stain before removing the insect from the dissecting dish for the preparation of a whole mount. In this way measurements were taken from tracheae left in their natural position with a minimum of distortion. Aqueous Bouin gave satisfactory results. Electron micrographs were taken at the Cavendish laboratory with a Siemens Elmiskop I electron microscope. For this purpose tracheae were fixed in 1% osmium tetroxide buffered at pH 7·4 and embedded in 1:1 butyl / methyl methacrylate.

Results

The epithelium and ecdysial membrane

The cells begin to divide on the 3rd to 4th day after feeding and continue to multiply until the 7th day. Nuclear destruction begins on the 4th to 5th day, reaches a peak by the 8th day, and is almost complete by the 9th day, when the new cuticle is apparent with the light microscope. The cytoplasm increases slightly in thickness with the increasing density of nuclei until the 7th day when the change is more abrupt. Fig. 1, A, B show the great activity of the cells at this time with abundant inclusions.

The slight withdrawal of the thickened epithelium which occurs from the 7th day is associated with the formation of what will be described as an ecdysial membrane, although some of its properties differ from the membrane described by Passoneau and Williams (1953) and Richards (1955). On the 7th day the inner cell-membrane appears thickened. By the 8th day the membrane appears in optical section as a thin PAS-positive line between the epithelium and the old trachea. At this stage it is not uniform: in surface view it is seen to be longitudinally striated (fig. 1, J). This is not an artifact of fixation, for the same striation is seen under phase contrast in fresh material. The reticulate nature of the membrane is well shown in sections under the

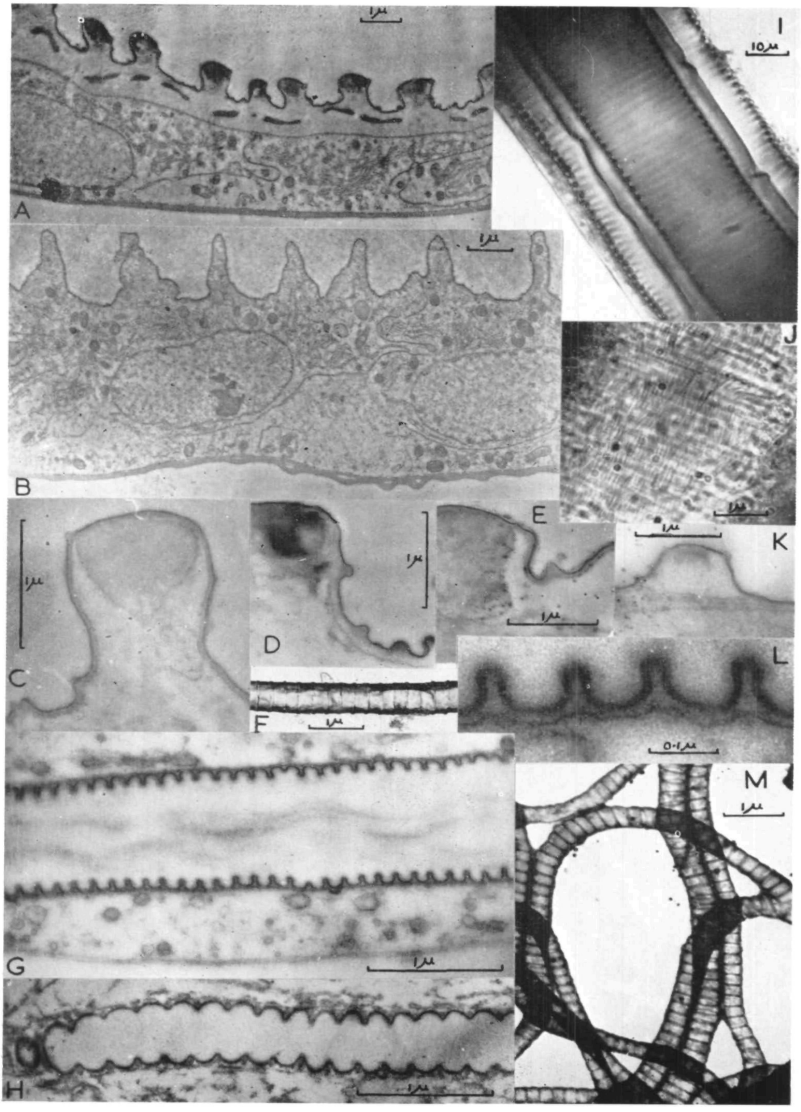

FIG. 1

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electron microscope (fig. 1, A). This striation persists until the new trachea has expanded some way from the old. It is most readily seen just as the new cuticle is buckling but it is certainly present before then. By the time the new trachea has reached its final diameter the ecdysial membrane is much more uniform (fig. 1, I). A sheet of it under the electron microscope appears without striations.

The membrane differs from ecdysial membranes described previously in that it has not been detected in the exuviae. It dissolves in the moulting fluid before ecdysis. Nor is it as resistant to strong alkali or diaphanol. Even after fixation it dissolves or is lost. It must be a different sort of carbohydrate from the chitin-protein complex of the outer layer of the lining membrane.

The pattern formed by the expansion of the lining membrane

When the ecdysial membrane has separated, the epithelium secretes the first layer of the new cuticle. By 8 to 9 days this layer shows up sharply in electron micrographs; it increases rapidly in area, buckling in the axis while the epithelium separates from the old trachea to take up the new diameter. Some phenomena may be interpreted as indicating that the epithelium is subjected to pressure from within at this stage. The equilibrium between the epithelium and the new cuticle appears to be upset in some preparations after fixation. The newly buckled cuticle takes up a position as if it were a broad helical spring forced to contract axially with asymmetry by a narrow central cord (fig. 2). It looks as if the expanding cuticle is forcing the epithelium to

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FIG. 1 (plate). All are electron micrographs except I and J, and all except D and E are of *Rhodnius*. All the electron micrographs are longitudinal sections except F and M, which are whole mounts.

A, 4th instar trachea 8 days after feeding. The ecdysial membrane appears as an interrupted line. The new cuticle is only just visible.

B, 9 days after feeding, showing the 5th instar trachea only. The new cuticle has just begun to buckle. The amplitude of the waves is slightly exaggerated by the oblique plane of the section.

C, single taenidium from a mature 5th instar trachea.

D, part of a taenidium and the epicuticle from a cockroach trachea.

E, the same for a mealworm larva. The epicuticle lining the inner face of the taenidium is similar to that between the taenidia but without the tubercles.

F, carbon replica of a tracheole from a 5th instar larva. The taenidia stand out as raised ridges.

G, a tracheole during its formation in the testis membrane of a 4th instar larva, 10 days after feeding. The tracheoblast cell-membrane lies along the bottom of the picture.

H, a mature tracheole from the same situation.

I, optical section with a light microscope of a trachea from a 4th instar larva, 12 days after feeding, showing the old and new tracheae and between them the ecdysial membrane.

J, Surface view of a 4th instar trachea, 8 days after feeding, focused between the nuclei and the old trachea. The ecdysial membrane appears as a series of striations diagonally from the bottom left of the picture at right angles to the taenidia.

K, 5th instar trachea, showing the formation of a taenidium. The cell-membrane has withdrawn from the inflated fold and material is gathering in the flattening tip.

L, a small part of G greatly enlarged to show the sheath-membrane round the buckling lining.

M, tracheoles showing the normal taenidial frequency.

take up the increased diameter of the new trachea. This is the increase in area of the cuticle to which Wigglesworth (1954) refers. The expansion is equal in the axis and the circumference. The outline of the cuticle seen in optical section was drawn with a camera lucida in a number of preparations of large

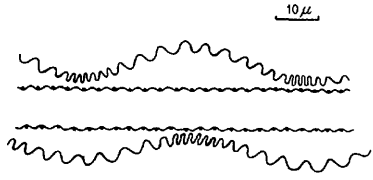

FIG. 2. The outline of the old 4th and new 5th instar tracheal cuticle in a larva 10 days after feeding, when the equilibrium between the new cuticle and the epithelium has been upset by poor fixation.

tracheae which show various stages of buckling. The axial expansion was then measured on the drawing with a cyclometer. Fig. 3 shows these data plotted against the increase in diameter. There is very good agreement with the hypothesis that the cuticle is expanding uniformly in the axis and the circumference.

Many authors have referred to the folding of the new cuticle (Wigglesworth, 1933; Wolfe, 1954), but the concept of buckling in response to stress has not

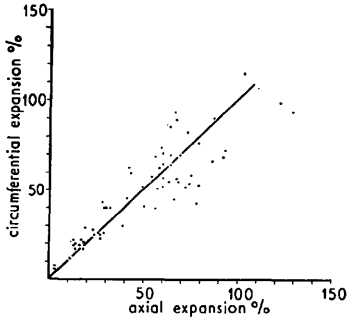

FIG. 3. The uniform expansion of the new tracheal cuticle in the length and circumference of the tube.

previously been introduced. The uniform expansion of the tracheal cuticle and the simple regular nature of the resulting deformation suggest that the cuticle is isotropic at this stage. The cuticle is also approximately constant in thickness in tracheae of different size and it is probably elastic (the completed two-layered membrane in the exuviae tends to lose the taenidial folds and the

isolated cuticulin layer alone shows scarcely any deformation). It is therefore reasonable to suppose that the newly formed cuticular tubes of the tracheae can be treated as thin-walled elastic cylinders with uniform properties varying only in radius. The tracheal cells also form a layer approximately uniform in thickness without any obvious anisotropy. Little is known about the elasticity

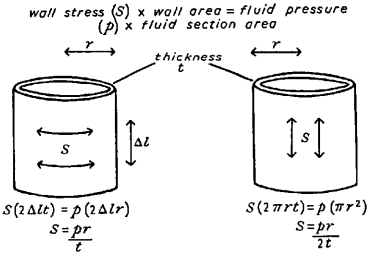

FIG. 4. Diagram showing how in a cylinder under uniform internal pressure the hoop stress is twice the axial stress. (After Castle, 1937.)

of such cells but it is probably low (Mitchison and Swann, 1954). The problem is to account for the buckling produced in the inner elastic cylinder when it expands against the outer one.

Now in a cylinder under uniform internal pressure the hoop stress is twice the axial stress (Timoshenko, 1936, p. 479). This was brought to the notice of biologists by Castle (1937). A cylindrical boiler blows up by tearing along the axis rather than by blowing off its ends (Fig. 4). The cuticle expands uniformly. Thus the restraining epithelium will tend to give first in the circumference. It need not give at all axially unless the critical axial stress for deformation in the cuticle exceeds twice the critical circumferential stress in the epithelium, Thus by balancing the critical strength of the cuticle between the critical stresses for axial and circumferential deformation of the epithelium, axial buckling alone could be produced. It may not be legitimate to refer to critical stresses in an epithelial layer in this way. The source of restraint is unknown. It is not likely that the old trachea embedded in the gel of moulting fluid contributes to the axial restraint since tracheae not forming round existing tracheae have a normal structure. The restraint may reside partly in the inner cell-membrane although this is unlikely since the membrane at first follows the buckles (fig. 1, B). For convenience the whole epithelium has been referred to as the source of the restraint since it appears to be the expanding cuticle which causes it to increase in diameter. The ultrathin cuticle might seem too fragile to move an epithelial layer 100 times as thick, but once axial buckling has occurred its resistance to lateral deformation increases. It may then be compared to a cylinder with stiffened rings. A knowledge of the Young's modulus of the epithelium would not be useful without information about its variation with time and stress. A layer of this sort might be markedly elastic

to sudden deformation, but over the hours or days of tracheal formation its behaviour might be almost plastic.

From Mitchison and Swann's work (1954) it is unlikely that cell-membranes respond linearly to stress. If these membranes and the whole epithelium did respond linearly over a wide range, then some axial extension of the cuticle should be observed before buckling. This is not detectable in practice. Buckling takes place almost as soon as the cuticle can be observed to be separate from the ecdysial membrane. This also indicates that the cuticle is appreciably elastic.

If the epithelial restraint had no yield point below the critical stress for circumferential deformation of the cuticle, then the system would be comparable to a cuticular tube under uniform external pressure (see Timoshenko, 1936, p. 479). Under these conditions the tube deforms asymmetrically, the number of lateral lobes depending on the ratios of length and wall thickness to diameter. Tracheae have not been reported with this deformation, but a slightly less regular buckling of this type is found on the nodes (junctions between tracheae from different regions). This pattern may be simulated by lining a glass capillary tube with rubber latex and causing it to swell in xylene. Edwards and others (1954) note but do not figure a tracheole with crossed helical thickenings. This also might well result from a restraint too great for the cuticle.

Whatever the critical stress and the precise origin of the restraint, the result in normal tracheae is buckling symmetrical with respect to the axis (helices are discussed latter). This would be expected from the foregoing discussion.

The frequency of buckling

Buckling in a cylinder which is expanding under axial restraint should be comparable to buckling in a cylinder under uniform axial compression (fig. 5). Symmetrical buckling would be expected in biological material with a low Young's modulus. In symmetrical buckling the buckling frequency, n, is related to the initial radius r, the wall thickness t, and Poisson's ratio (v) for the material, in the expression

$$1/n = \frac{\pi\sqrt{(rt)}}{\sqrt[4]{\{12(1-v^2)\}}} \quad \text{(Timoshenko, 1936, p. 441).} \qquad (1)$$

Poisson's ratio (v) must lie between $+\frac{1}{2}$ and -1. v for steel $= 0{\cdot}3$, and for rubber $v = 0{\cdot}46$–$0{\cdot}49$ (Kaye and Laby, 1948), so for biological materials v should be close to $0{\cdot}5$.

For $v = 0{\cdot}3$, $\quad \frac{\pi}{\sqrt[4]{\{12(1-v^2)\}}} = 1{\cdot}72,$

and $v = 0{\cdot}5$, $\quad \frac{\pi}{\sqrt[4]{\{12(1-v^2)\}}} = 1{\cdot}81.$

Thus there should be very little error in simplifying equation (1) above to

$$1/n = 1{\cdot}8\sqrt{(rt)}. \qquad (2)$$

Now if the formation of taenidial folds is comparable to buckling, it should be possible to describe it by this formula. n, the number of half-waves of buckling per unit length, is known and by using preparations similar to fig. 1, I, the initial radius (r) may also be measured. If log r is plotted against log n for tracheae of different size, the result should be a straight line of slope $-\frac{1}{2}$, the position of the line being fixed by the value of t, the initial thickness. Fig. 6, A shows such a graph for *Rhodnius* 5th instar tracheae taken just before emergence from the 4th instar exuvium. The slope is a fair approximation to $-\frac{1}{2}$. Fig. 6, B, C, D gives similar graphs for tracheae from intermoult 3rd and 4th instar *Rhodnius* and from the nymphal cockroach, *Periplaneta*. r here is the final radius of the trachea, but since the increase in diameter is not a function of the radius this should not have altered the slope. These graphs also show a good approximation to the slope predicted by theory.

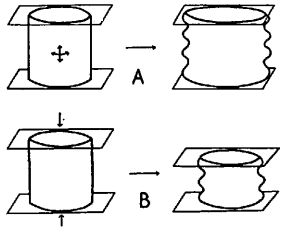

FIG. 5. The formation of buckles in an expanding cylinder restrained in the axis (A) should be comparable to buckling in a cylinder under uniform axial compression (B).

In fig. 6, A, C, D the slope is slightly less than $-\frac{1}{2}$. This error would occur if the taenidia were farther apart than theory predicted by an amount inversely proportional to r. Now the buckling frequency has been measured upon tracheae with completely formed taenidia. It was assumed with Keister (1948) that the spacing of taenidia did not change during their formation, but this is probably not quite correct. In some tracheae the amplitude of the waves is so large that the original buckles must have been forced apart as the cuticle continued to expand. In tracheae from the testis which increase in diameter by up to 300% this has certainly happened. This axial expansion after buckling will tend to be inversely proportional to the radius, since a small trachea will have relatively thicker walls and will be proportionally resistant to compression.

If this interpretation is correct, data taken from newly buckled tracheae, or from tracheae which have increased little in diameter, should show an even closer approximation to the theoretical slope of $-\frac{1}{2}$ than fig. 6, A–D. Accordingly the buckling frequency was measured on tracheae before taenidial formation was complete. Fig 6 E shows that the buckling frequency is then very close to the theoretical indeed. The amplitude of buckling waves is also very small in intermoult *Tenebrio* larvae. Fig. 6, F shows that here also the slope is close to that predicted by theory.

Thus as far as the slope of the graphs is concerned there is a very plausible agreement with the theory that taenidia result from buckling of the cuticle. The data are summarized in table 1.

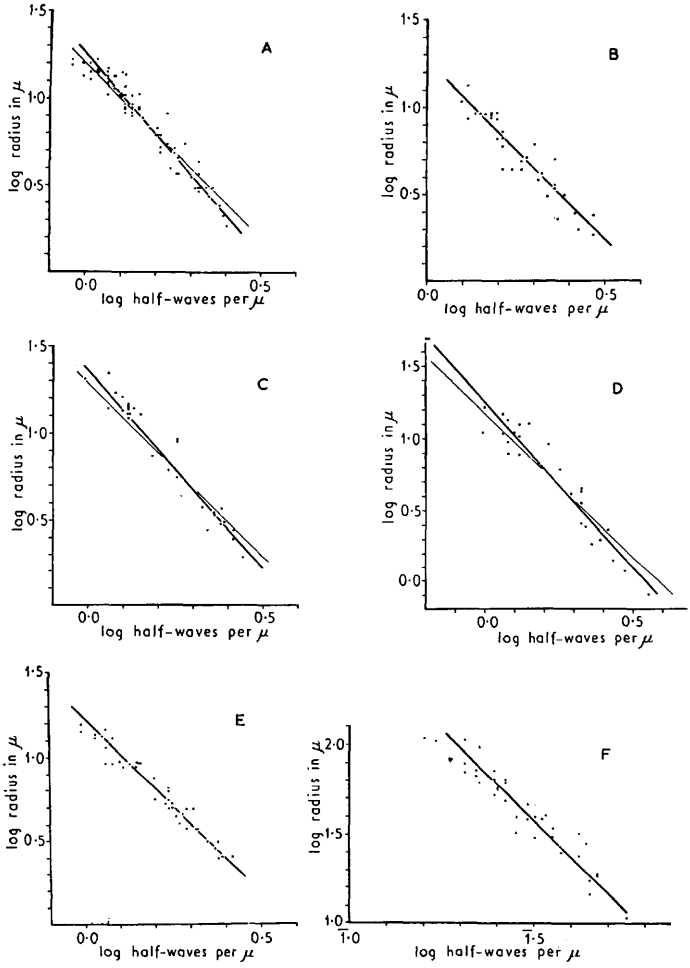

FIG. 6. The relation between taenidial frequency and the radius of tracheae. A, 4th instar *Rhodnius* larvae just before moulting to the 5th instar. B, 3rd instar *Rhodnius*. C, 4th instar *Rhodnius*. D, nymphal cockroach. E, 4th instar *Rhodnius* larvae, 10 days after feeding, before expansion of the new 5th instar trachea is complete. F, *Tenebrio* larvae. Ordinate: B, C, D, F, log radius of the trachea; A, E, log radius of the old trachea. Abscissa: B, C, D, F, log number of taenidia per 2 μ; A, log number of taenidia per 2 μ in the new 5th instar trachea; E, log half-waves of buckling per 1 μ in the new 5th instar trachea. A slope of $-\frac{1}{2}$ is indicated by a thin line where this deviates sufficiently from the best-fitting straight line for the data.

The thickness of the cuticle

Since the thickness t is proportional to $(1/n)^2$, quite small errors in the measurement of the spacing of the taenidia will have a marked effect upon estimates of the thickness. Such errors might tend to affect tracheae of all sizes equally so that the fit of the slope is not necessarily an indication of the accuracy of the estimate of thickness. The standard deviations in table 1 indicate the precision of the agreement with the slope of $-\frac{1}{2}$ rather than the accuracy of the thickness estimate.

TABLE 1

Figure	*Data from*	*Correlation coefficient*	*Slope*	*Thickness and S.D.*	*Diameter increase*	*Probable diameter increase*	*Thickness and S.D.*
				(Å)	(%)	(%)	(Å)
5, A	5th instar *Rhodnius*, taenidia before ecdysis	−0·95	−0·43	197 34	67	..	..
5, E	5th instar *Rhodnius*, newly buckled tracheae	−0·97	−0·48	196 27	48	..	..
5, F	Intermoult *Tenebrio*	−0·83	−0·48	..	..	20	1,000 205
5, B	Intermoult 3rd instar *Rhodnius*	−0·94	−0·49	..	..	50	266 50
5, D	Intermoult *Periplaneta*	−0·97	−0·43	..	..	50	311 90
5, C	Intermoult 4th instar *Rhodnius*	−0·97	−0·44	..	..	50	239 50

The initial thickness of the cuticle calculated from the data in fig. 6, E is 196 Å, S.D. 27 Å. This estimate has been verified approximately with the electron microscope. Preparations before buckling have not been used for this since they may not have reached their final thickness preparatory to expansion. Fig. 1, B shows a large trachea which has just begun to buckle. The amplitude of the buckles is exaggerated in this preparation because the section does not pass exactly down the meridian of the tube. Measurements of the thickness of the cuticle at this stage lie between 160 and 200 Å. The most precise measurements from enlarged plates taken at a magnification of 20,000 suggest a thickness of about 180 Å. This is very close to the predicted value. The cell-membrane follows the buckled cuticle and perhaps contributes to the calculated thickness. This would account for the slightly lower value of the

measurement, but it is not possible from the photographs available to say that the measured thickness differs significantly from that calculated. Most of the other errors tend to make the calculated thickness maximal. The wider spacing of taenidia than first-formed buckles has already been mentioned. The magnitude of the initial circumferential restraint is unknown but it would also tend to depress the buckling frequency.

Calculations of thickness from the data in fig. 6, B, C, D, F would tend to exaggerate these errors since tracheae elongate slightly upon release from the exuvium. These data are also less reliable, since a value for the initial radius has to be assumed for the calculation. A poor estimate for this can be made from the amplitude of the folds, but this is not reliable since it will be affected by the extension of the trachea at ecdysis. The figures in the last column in table 1 are therefore included only to show that they do not greatly conflict with the more carefully obtained data for 5th instar *Rhodnius*. All values are high, as expected. It is perhaps significant that electron micrographs of mature tracheae of *Tenebrio* and *Periplaneta* show a thicker cuticle than 4th or 5th instar *Rhodnius* (fig. 1, D, E).

The formation of helices

The previous argument has been simplified by treating the low-pitch helices which occur in large tracheae as if they were annuli. It seemed likely, as Richards (1951) suggested, that a tangential shearing force added to the forces responsible for annular taenidial formation might cause a helix to form. The critical normal stress A_{cr} necessary to produce annular buckling from axial compression is given by

$$A_{cr} = \frac{Et}{r\sqrt{\{3(1-v^2)\}}} \quad \text{(Timoshenko, 1936, p. 441)}, \tag{3}$$

where E = Young's modulus and v = Poisson's ratio for the material of the tube-wall of thickness t and radius r. Putting in reasonable values for v ($= 0{\cdot}48$), t ($= 0{\cdot}02\,\mu$), and r ($= 20\,\mu$), this becomes

$$A_{cr} = \frac{0{\cdot}02E}{20\sqrt{\{3(1-0{\cdot}48^2)\}}} = \frac{0{\cdot}02E}{30{\cdot}4} = 7\times10^{-4}E.$$

The critical shear necessary to produce helical buckling is given by

$$T_{cr} = \frac{E}{3\sqrt{2}(1-v^2)^{\frac{1}{2}}}(t/r)^{\frac{3}{2}} \quad \text{(Timoshenko, 1936, p. 486)}. \tag{4}$$

Putting in the same values as before this becomes

$$T_{cr} = \frac{E}{3\sqrt{2}(1-0{\cdot}48^2)^{\frac{1}{2}}}(0{\cdot}02/20)^{\frac{3}{2}} = \frac{E}{3{\cdot}49}\times3{\cdot}2\times10^{-5} = 9\times10^{-6}E.$$

Thus the critical shearing stress necessary to produce helical buckling is only a very small fraction of the axial stress necessary to produce annular buckling. Helical folds in tracheae are not comparable to those produced by

torsion alone, for this induces helices with a high pitch with lobes in the circumference rather than the axis. The low-pitched spirals of tracheae probably result from an axial stress with only a slight shear component. This may be illustrated by a model. A thin rubber sleeve can be prepared by allowing rubber latex to dry over a glass rod. If this is lubricated with water, it will slide and fold freely on the rod. Axial compression on the rubber induces the formation of annuli, while twisting produces helices. Compression with a very slight twist produces helices with about the same pitch as are found in tracheae.

Thus the torsion necessary to change annuli into helices is very small. It seems probable that randomly-occurring torsional stresses in the tissues could be responsible. The distribution of helices in tubes of different diameter supports this. Whereas the critical axial stress is proportional to the ratio of wall thickness to radius, the critical shear stress is proportional to this ratio[8]. Thus while the critical shear may be only just over 1% of the critical axial stress in tubes of radius 20 μ, it is over 8% in tubes of radius 1 μ. Small tubes are almost always annular and large tubes almost always helical.

The control of expansion

It will be shown in a future paper that adjacent parts of a trachea may increase in diameter by different amounts. Thus the agent controlling expansion is unlikely to reside in the moulting fluid. Either the extent of expansion is intrinsic in the membrane when first formed and is released to completion (perhaps by the moulting 'fluid'—Passoneau and Williams (1953) found that the composition of the 'fluid' changes with time; initially it contains tyrosinase, for example), or the expansion is controlled through the epithelium. If the former hypothesis were correct, then it might be expected that the initial thickness of the membrane would vary with its future diameter. There is some evidence that this is so. If the initial thickness calculated from measurements of taenidial frequency and radius as in fig. 6, A and table 1 is plotted against the increase in diameter, there is a significant positive correlation. But this could be and probably is the result of the poor fit for slope caused by the slight axial expansion during taenidial formation. This criticism could be discounted to some extent if the thickness calculated from mature tracheae with a very small increase in diameter proved to be significantly less than that calculated (fig. 6, E) and approximately verified with the electron microscope for normal tracheae.

Fourth instar larvae were induced to moult without feeding by joining in artificial parabiosis with fed larvae (Wigglesworth, 1934). Preparations were made as in fig. 1, I. The taenidia are fully formed but the amplitude of the waves is commensurate with the small increase in diameter (mean = 35%). The thickness calculated from these tracheae is only 162 Å, S.D. 47 Å, which is significantly lower than normal (P = less than 0·1%). Full weight should not be given to this result because of the small change in taenidial frequency which it represents, but it is suggestive. Now these preparations are highly abnormal. An insect forced to moult without adequate reserves might very

well lay down thinner membranes whether or not the future diameter depended on it. It is unwise to infer that the initial membrane thickness is proportional to the future diameter in the normal variations in growth. Nevertheless, the result does not conflict with the hypothesis that the future diameter is latent in the first formed membrane.

The formation of the taenidia

The spacing of taenidia in tracheae of different size depends upon the buckling of the cuticle already considered. The size of the taenidia depends

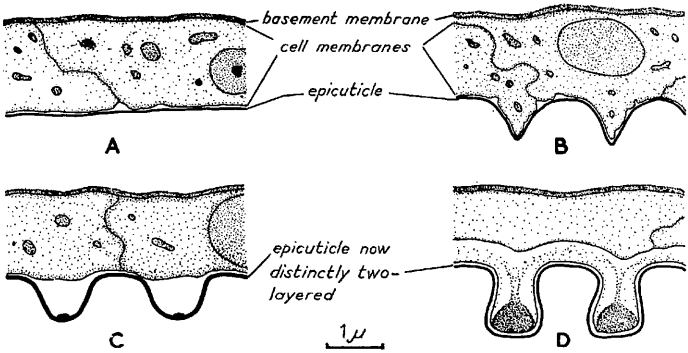

FIG. 7. Diagram showing the formation of the taenidia as seen in longitudinal section. A, the cuticulin is smooth as in fig. 1, A. B, it has begun to buckle as in fig. 1, B. C, the folds have inflated and the apices of the buckles have flattened with the deposition of the new taenidia as in fig. 1, K. D, the appearance of mature taenidia with flattened inner faces as in fig. 1, C.

upon the expansion. A series of large tracheae showing a range of increases in diameter was prepared by feeding the larvae to a varying extent. From these it was clear that the amplitude of the waves in mature tracheae varied with the diameter increase.

Fig. 1, A, B, K, C shows the progress of buckling. When the trachea has almost reached its final diameter the folds inflate with the deposition of the taenidia. In the first-formed buckles the cell-membranes follow the line of the expanding cuticle (fig. 7, B), but as expansion proceeds the cell-membranes are withdrawn, leaving clear spaces in which the taenidia are deposited (fig. 7, C). Now the expansion of the circumference will tend to exert a torque upon elongated particles free to move in these spaces so that they tend to build up in an oriented band at the apex of the folds. The electron micrographs give the impression that this is happening. With further expansion more material is laid down or crystallizes out, tailing off towards the epithelium (fig. 7, D). The formation of tangentially oriented bands would be expected to exert a considerable restraint upon the cuticle expanding in the circumference. In agreement with this the tubercles which probably have

their origin in the tiny irregularities at first seen all over the cuticle, are 'ironed out' over the inner surface of the taenidia where it tends to be flattened rather than rounded.

The appearance of the taenidia and the orientation of their micelles would be expected from the secretion of an endocuticle over an expanding buckled surface.

The orientation of chitin micelles in the lining membrane

Long molecules in thin films become oriented in fibrils at right angles to a lateral pressure (Tachibana and others, 1955). Thus the axial orientation of chitin micelles in the tracheal cuticle could be the result of lateral pressure. The time when this occurs is not known, since neither the taenidia nor the lining membrane withstand treatment for chitin purification very well during the early stages of their formation. The new cuticle when first formed appears as a thin sharply defined electron-opaque line. This has not been resolved into a double layer as in a cell-membrane; if anything it appears granular. During expansion it becomes thicker and by the time the epithelium has withdrawn from the folds it is distinctly two-layered as in mature tracheae. The first-formed cuticle seems to be continuous in time with the lining cuticulin layer, the main change during expansion being the addition of the chitin-protein layer on the epithelial side. It is presumably during expansion that the orientation occurs. The tube of the newly formed cuticle is initially under the uniform pressure of the epithelium, but as soon as buckling has occurred the axial restraint will be released and the expanding layer exposed to lateral restraint alone. After buckling the critical stress for lateral deformation will rise, the properties of the tube then being equivalent to a cylinder stiffened with supporting rings. As expansion proceeds the lateral pressure may therefore increase above the axial pressure which initiated buckling without inducing further deformation. This will culminate in the restraint imposed by the newly formed taenidia which eliminates some of the tubercles. Thus if at some stage during expansion the tracheal cuticle contains long, unoriented particles, their final orientation should be axial.

Histochemical changes in the trachea during its formation

According to Wigglesworth (1947) the newly secreted cuticulin layer over the abdomen in *Rhodnius* gives a strong positive reaction with Millon's reagent. This is much less obvious in the tracheae. Perhaps this is due to its extreme thinness, but it may also be correlated with the lack of pigment. The new trachea remains almost Millon-negative until the 12th day, when the taenidia are fully formed, after which it reacts positively. Most of the colour appears to be in the lining membrane, but there is some in the taenidia.

Changes in PAS staining are the reverse of those given by the Millon test. At 8 days, before the secretion of the new cuticle, the epithelium stains strongly. So does the ecdysial membrane, the newly formed cuticle, and the newly formed taenidia on the 9th–10th days. By 12 days the completed trachea

almost ceases to stain; its very pale pink colour is similar to that in intermoult tracheae. By 13 days the ecdysial membrane has disappeared and the old taenidia stain strongly as they dissolve in the moulting fluid. It is perhaps as a result of attack by the moulting fluid that the tracheal exuviae are PAS-positive.

In theory chitin should be PAS-positive (Pearse, 1954), but Richards (1951, 1952) and Wigglesworth (1956, personal communication) record that some material which certainly contains a polysaccharide may not stain. Richards suggests that the carbohydrate in insect cuticles may be patent or masked. This seems to be so with the chitin in the lining membrane and taenidia of tracheae. The incorporation of Millon-positive material is associated with the tracheae becoming PAS-negative. Now chitin fails to give a positive PAS test if the glycol groups are substituted (Pearse, 1954), and this is what might be expected in an intimate chitin-protein association. In agreement with this Wigglesworth (1956) records that the endocuticle in *Rhodnius* becomes PAS-positive when it is attacked by the moulting fluid. The endocuticle in *Rhodnius* exuviae is almost Millon-negative, in sharp contrast to the rest of the exuvium, and gives a positive PAS test.

The masking of chitin in tracheae is different from that described by Richards. The masking by sclerotization in the bee is probably comparable to that in the abdominal epicuticle of *Rhodnius*. In exuviae this layer contains chitin but is PAS-negative, becoming positive after extraction with mild alkali. It seems probable that there may be at least three processes which interfere with the PAS test—the incorporation of protein in the taenidia, the tanning of the chitin-protein layer in the lining membrane of the trachea, and the tanning with lipid impregnation in the bee and the abdominal epicuticle of *Rhodnius*.

The structure and formation of tracheoles

Tracheoles are readily teased from the tergites of larval *Rhodnius* for whole-mount electron microscope preparations. They are blindly ending tubes with an annular or helical pattern on the walls (fig. 1, M), which shows up as a raised ridge in carbon-shadowed preparations (fig. 1, F). In section the structure is seen to be very simple. The lining membrane appears uniform and sharply delineated (90–120 Å thick) like the cuticulin layer on the tracheae (fig. 1, H). There is no chitin-protein layer and the taenidia are small and indistinct. The tracheole is enveloped in the cell-membrane and there may be traces of an inner membrane surrounding the tube. Superficially a tracheole resembles a trachea with a different buckling frequency. This suggested that the expansion and buckling hypothesis might be extended to account for tracheole formation.

With this in mind a number of measurements of radius and taenidial frequency were made on preparations similar to fig. 1, M in an attempt to obtain graphs for tracheoles similar to those in fig. 6 for tracheae. The results were not encouraging. The calculated initial thickness was never less than

200 Å, whereas the final thickness observable in photographs is not more than 120 Å. Similar results were obtained from sections and other published figures of tracheoles. If the expansion and buckling hypothesis were to be retained, this could only be explained by assuming that the tracheoles had extended in length after their formation. This would be expected from Wigglesworth's work on tracheole migration. There was a further difficulty in applying the hypothesis to tracheole formation. In tracheae there is an epithelial layer, some part of which could act as a restraint to the expanding cuticle. In tracheoles with a lining membrane not greatly thinner no structure had been found which could act as a restraint apart from the tracheoblast cell-membrane.

Both these difficulties were resolved by sectioning tracheoles during their formation. There is much growth of the testis and its tracheal supply during the 4th–5th moult. Testis membranes were sectioned 7–12 days after feeding. On the 10th day tracheoles were seen in the stage shown in fig. 1, G, L. The lining looks much as a buckled membrane might be expected to look, and on its outside is a second unbuckled membrane. It seems plausible to suppose that this second sheath-membrane is acting as the restraint to expansion of the inner lining. Assuming that expansion has taken place with little increase in tube length, the initial radius of this tube may be estimated from the amplitude of the waves. Using this estimate (diameter increase = 77%), the initial thickness calculated from expression (2) comes out to be about 46 Å. Therefore with a diameter increase of 77%, if expansion is accompanied by swelling, the final thickness should be about 82 Å. The measured thickness is about 85 Å. Several other preparations have a similar buckling frequency in this early stage of formation. These estimates are only approximate and few preparations appear as reliable as fig. 1, G, but a better agreement with the expansion and buckling theory could not be expected.

Discussion

The ecdysial membrane

This membrane was first seen in the fully formed condition (fig. 1, I) and it was then thought that it might correspond to an endocuticle comparable to that laid down over the rest of the body after feeding (Zwicky and Wigglesworth, 1956). The positive PAS test supported this, since Wigglesworth (1956) records a strong positive PAS for endocuticle attacked by the moulting fluid. But its appearance and situation when newly formed suggest that it is the homologue of the ecdysial membrane containing chitin described by Passoneau and Williams (1953) and Richards (1955). The loss of the membrane in the moulting fluid and after treatments for purifying chitin contrasts with Richards's ecdysial membrane, which contains chitin and is not attacked by the moulting fluid. The longitudinal striations in the newly formed membrane are a puzzling feature which shows that a coherent membrane may be formed without the agency of a cell surface. Richards (1955) figures a pleated pattern in an ecdysial membrane from the 'face' of an exuvium of a *Cecropia*

moth pupa which has a pattern not unlike fig. 1, J, but this is in a mature ecdysial membrane. The similarities between the membrane in tracheae, the ecdysial membrane of the American authors and Wigglesworth's post-feeding cuticle over the *Rhodnius* abdomen, suggest that they may all be variants of the same thing. Whatever the nature and function of the ecdysial membrane, it is unlikely that it plays any part in determining tracheal structure for there is no ecdysial membrane in tracheae not formed round existing tracheae.

Buckling and micelle orientation

The precision with which a description in physical terms fits a biological event is the only criterion of validity, but a good fit does not establish a causal sequence. Nevertheless, the simplicity and regularity of the deformation in the tracheal cuticle has allowed the hypothesis of expansion and buckling to be verified in a way which would be extremely difficult for many of the surface cuticular patterns—although these may occur in the same way. For example, the stellate pattern on the abdomen of larval *Rhodnius* described by Wigglesworth (1947) may be imitated by causing a thin film of rubber on a glass surface to swell with xylene.

If the interpretations of tracheal and tracheole formation given here are correct, they present a remarkable example of organic design (Pantin, 1951). An identical 'trick' has been used to form tubes with similar functional requirements at two different levels of organization. This leads to a real distinction between tracheae and tracheoles, in terms of the thickness of the lining and hence the buckling frequency, the structure responsible for the restraint, and perhaps the mechanism of expansion.

The orientation of chitin micelles by stress is now well established in insects (Fraenkel and Rudall, 1947; Lees and Picken, 1945; Picken, Pryor, and Swann, 1947). The taenidia are another example of orientation which must take place away from a cell surface. In the development of lepidopteran scales the reverse procedure may occur (Picken, 1949). Here the growth of long fibrillar aggregates may cause the elongation of the scales. The sensory hairs over the abdomen of *Rhodnius* also contain chitin oriented in the axis and they are probably protected by a cuticulin layer. The possibility should be considered that it may have a role in their formation.

The mechanism of expansion

Very little can be said about the mechanism until more is known about the chemistry of the cuticle. In tracheoles, expansion is probably the result of swelling. In tracheae also the first-formed layer gets thicker, losing its distinctness on the epithelial side, its place being taken by the chitin-protein layer. On the other hand, it is an unusual sort of swelling which allows the inner cuticulin membrane of the trachea to appear much the same during and after expansion by up to 300%. It seems to be a property of this layer to maintain an approximately constant thickness; it is remarkably similar in all cuticles examined, the variable factor being the chitin-protein layer on the

epithelial side. The total thickness (360–570 Å) of the cuticle in a fully formed trachea is more than twice the observed or calculated initial thickness, so that something more complex occurs during tracheal formation than the swelling of a single membrane. The extra thickness may be due to the incorporation of Millon-positive material when the membrane is stabilized by tanning 12 days after feeding.

Cuticle terminology applied to tracheae

The epicuticle is normally defined as the outer non-chitinous part of the cuticle. This would restrict it to the cuticulin and morphologically outer layers excluding the chitin-protein layer, with which it is intimately associated in both tracheae and surface cuticle (Locke, 1957). This would seem to be an artificial definition. If the term epicuticle is to be useful, it should include the chitin-protein layer. Terminology is not important where the structure is well understood, but the separation of the cuticulin and chitin-protein layers may tend to obscure what may be the most fundamental feature in cuticle development, the orientation of micelles by the expanding cuticulin.

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