An investigation of the structure and function of the peritrophic membrane of the American cockroach, Periplaneta americana, with special reference to the possible effects of tannins on water movement and permeability of dyes through the peritrophic membrane.

by

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DECLARATION

I declare that the material contained in this thesis is my own work, unless otherwise acknowledged, and it has not previously been submitted for consideration as part of any academic award. I consent to photocopying of the thesis, conditional on award of the degree.

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SUMMARY

The peritrophic membrane (PM) is often said to protect the midgut epithelium from abrasion by solid food particles, or act as a physical barrier to penetration of the midgut epithelium by micro-organisms, or as an ultrafilter. Recently, it has also been widely implicated in the conservation of enzymes by creating an ectoperitrophic space next to the midgut epithelium which allows a flow of solutes, including enzymes, counter to the movement of the ingesta, and concentrates these solutes in the caeca. Other work has also implicated the PM of polyphagous phytophagous insects in protection of the midgut from ingested tannins. The PM in orthopteroid insects (including Periplaneta americana) is widely regarded as being "delaminated" from the entire midgut.

The present study briefly reviews this work and investigates the structure, function and permeability of the PM in P. americana and the possible effects tannins may have on these parameters.

P. americana was used especially because it is a generalist feeder not secifically adapted to dietary tannins; limited comparisons were made with another species of cockroach, Panesthia cribrata which might encounter tannins due to its wood-eating habit.

Pulse radiolabelling experiments, feeding experiments with coloured diets, and injection of dyes into the abdomen indicated that the bulk of the PM is secreted by cells of the anterior end of the midgut. When a previously fasted roach began to feed a new PM was produced at 5-7 mm/hr and production of the PM could possibly also be influenced by some humoral stimuli.

Feeding experiments and injection of dyes also showed retention of dyes in the caeca, perhaps due to a countercurrent system and/or to other physical concommitants of the interaction of the PM and midgut, including patterns of compaction of food and changing permeability of the PM after it is secreted.

Polyphenols affected the PM in P. americana differently in vitro and in vivo: when PM sections were soaked in solutions of tannate or gallate, polyphenolic depositions could be observed only in acidic conditions. When cockroaches ingested polyphenols in a standard diet containing only starch, sugar and silica, no polyphenolic depositions were seen on the PM although the PM structure was affected. Tannic acid in their diet suppressed consumption and other feeding parameters of the insects but no significant effect of tannic acid on permeability parameters of the PM could be demonstrated.

Electron microscopy of the PM in P. americana showed it to be a variform lamellate cylindrical sheath holding the solid

to be a variform lamellate cylindrical sheath holding the solid ingesta. The layers consisted mainly of hexagonally or orthogonally orientated grid systems. The ectoperitrophic surface, however, was differentiated along its length: where it overlaid the oesophageal invagination, the microfibrils appeared disorganised, perhaps reflecting the apparent "fluidity" of the PM in this region. Here also, the PM appeared closely associated with the oesophageal invagination due to numerous small spines, or microtrichia. Posterior to this "fluid" area is a region of hexagonal and/or orthogonal grid systems. From 2-3 mm past the tip of the oesophageal invagination and for the rest of the length of the midgut, these grid systems were overlaid with a largely amorphous ectoperitrophic surface layer, consisting of matted fibrils that were closely associated with the underlying grid system and had their interstices filled with a granular substance (hence the amorphous appearance of the layer). It was this ectoperitrophic surface that was affected by ingested tannic acid which caused an apparent lack of the granular substance, leaving only the matted fibrillar network at the surface.

The hydraulic conductivity of the PM in P. americana was found to be 0.091 cm/s.bar by an in vitro blind-sac perfusion technique. Using the same technique, permeability to various dyes was carried out when PM sections were bathed in water. Three charged dyes of formula weight 300 to ca. 1300 Dalton, Azure B, Trypan blue, and Alcian blue, all increased the

hydraulic conductivity, but a protein-dye conjugate, Azoalbumin, with a formula weight of ca. 7×10^4 Dalton, caused an effective blocking of membrane pores and the hydraulic conductivity was reduced to 0.006 cm/s.bar. When the PM sections were bathed in saline, this apparent blockage of the PM was almost totally removed with a ten-fold increase in hydraulic conductivity. In the absence of Azoalbumin, any combination of saline/water either side of the membrane increased hydraulic conductivity by about 150%. Moreover, the effects of the saline were reversible. These results suggest that the PM acts as a microfilter, and as an ultrafilter under some conditions. As a result, solutes and perhaps enzymes, can remain in the midgut for much longer periods than initial passage of particulate ingesta might suggest. It is proposed that the PM subserves digestive processes in a variety of ways, based on its retention and compaction of solid particles within its lumen.

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CHAPTER 1 : General Introduction.

1.1 Function of the Peritrophic Membrane (PM).

The PM is often said to prevent abrasion of the midgut epithelium from solid food particles (e.g. Wigglesworth, 1972). Its absence in some solid feeders and presence in some liquid feeders (see e.g. House, 1975), suggests that there are other functions of the PM, however. For example, it could act as a barrier to micro-organisms (Andries, 1982), as an ultrafilter (Peters, 1976) or microfilter (House, 1975). It may also prevent rupturing of the midgut in dipterans (see Peters, 1976) or prevent food particles entering the caeca (Richards and Richards, 1977). The PM also has more specific functions such as its use in the formation of cocoons (see Richards and Richards, 1977) and its involvement in the excretion of insecticide (see Peters, 1976).

Perhaps the most significant general effect of the PM is that it creates two spaces within the midgut, the ectoperitrophic space (i.e. the space between the midgut and the PM) and the endoperitrophic space (the lumen of the PM). In fasting locusts this allows what Dow (1981a) describes as a countercurrent system to operate, where fluid derived from the Malpighian tubules moves anteriad while the contents of the PM move "counter" to this, i.e posteriad; it has been suggested

that such a system serves to conserve enzymes and nutrients.

Terra and co-workers have suggested that the PM, as well as allowing a countercurrent system, separates two extracellular sites of digestion as an adaptation to conserve secreted enzymes (Terra and Ferriera, 1981). They have found, for instance, in two sciarid species (Rhynchosciara americana and Trichosia pubescens), that enzymes involved in the initial metabolism of food molecules penetrate the anterior PM into the endoperitrophic space, and then cross back again more posteriorly when the polymers they hydrolyze are small enough to accompany the enzyme (Terra, et al., 1979). The resulting oligomers and hydrolases are said to be moved by a countercurrent flux to the caeca in the ectoperitrophic space (Ferriera, et al., 1981). Enzymes involved in this further stage of digestion in the ectoperitrophic space and caecal lumina are presumed to be too large to penetrate the PM or are thought to be bound to the plasma membrane (Terra et al., 1979, Terra and Ferriera, 1983). The cassava hornworm (Erinnyis ello) also appears to have the same basic features of spatial organisation of digestion as described above for the two sciarids (Santos et al., 1983; Santos and Terra, 1984).

Peters (1976) suggests that since multilayered PMs often have an asymmetric distribution of carbohydrates, they could be compared with epithelial surface coats. The formation of the PM also suggests that it could be a hypertrophied and constantly

detached surface coat and therefore may have properties similar to those of a "glycocalyx" (Peters, 1982). That it might do so is suggested by the finding of lectins with high specificity to mannose on the endoperitrophic surface of the PM of Calliphora erythrocephalla (Peters et al., 1983). Aminopeptidases were also found as immobilised enzymes adsorbed onto the PMs from insects of a wide range of insect orders (Peters and Kalnins, 1985).

Bernays and Chamberlain (1980) have shown that PMs from a polyphagous acridid tolerant of ingested tannic acid could adsorb 20% of the ingested tannic acid while polyphagous acridids generally have more substantial PMs (in Bernays, 1982 - unpublished data).

PMs therefore appear to have diverse functions or effects that may be highly specialised in some insects (e.g. in cocoon formation) or have more general applications in others (e.g. providing the structural framework for a countercurrent system and spatial separation of intermediate and final stages of digestion).

1.2 **Structure and production of the PM.**

In most insects the entire midgut epithelium is said to produce the PM (Type I production) while in some, the PM is produced from a band of cells at the anterior of the midgut

(Type II production, especially well known for Diptera). Richards and Richards (1977) claim that a rigorous distinction of a Type I PM by delamination from a Type II PM is not warranted since both are in effect, due to secretion. They also suggest that on the basis of the data available, secretion of the PM seems to follow a pattern similar to structural secretion by vertebrate cells (e.g. work by Pallade and co-workers referred to by Richards and Richards, 1977), but with some modifications. Precursors are synthesised by ribosomes on a well developed endoplasmic reticulum (ER); the product is segregated into cisternae of the ER and is transported intracellularly to Golgi-like areas, which may have defnite Golgi characteristics but at other times (or in some species) are more amorphous. Without apparent storage in vesicles, the secretion is passed out of the cell as a finely granular product in which microfibrils appear and aggregate as post-secretion phenomena; the aggregation into microfibrils and then into larger microfibres may be far removed from cell surfaces. Richards and Richards stress the need for appropiate tracer tests, however, if the above synopsis is to be confirmed.

Richards and Richards report both Type I and II modes of production occurring in several orders of insects, and also other anomalous types of production. They suggest that a more useful functional distinction might be made on the basis of whether the PM is continuously produced or in response to certain stimuli (e.g. extension of the midgut).

A general description of PMs could be that they are a variform, multilamellate sheath surrounding the ingesta (solid or fluid) in the midgut and sometimes hindgut, and whose components are derived from cells of the midgut. They normally contain chitin that is closely associated with protein and forms microfibrils (Richards and Richards, 1977). The "chitin" microfibrils are considered to be the structural elements of the PM and it might be these that act as a barrier to micro-organisms (Peters, 1976). The layers of the PM can appear granular or colloidal in nature, or have the microfibrils aggregated into fibrous mats, or orientated 60 or 90 degrees to each other to give hexagonal and orthogonal grid meshworks respectively (Peters, 1976; Richards and Richards, 1977).

1.3 **Overview of the present investigation.**

This study investigates the structure, function and the permeability of the PM in Periplaneta americana and the possible effects that tannins may have on these parameters.

P. americana was used especially because it was a generalist feeder not specifically adapted to tannins. Limited comparisons were made with another cockroach species, Panethesia cribrata, which might encounter tannins due to its wood-eating habit, and with some other insect species, throughout the study.

In recently moulted cockroaches, the PM is highly compacted in the posterior region of the midgut (the total length of the PM in the midgut in such individuals can be twice the length of the midgut) and is darkly pigmented, grading from brown to almost black (normally it is relatively unpigmented and translucent). It is suggested (Chapter 2) that it may have a function at ecdysis in the adsorption of moulting fluid.

Sections of PM from various parts of the midgut when subjected to tensile stress had a mean per cent extension from 15 - 17% with a maximum breaking load of 1.20 - 1.26 g (Chapter 2). The ability for the PM to undergo such an extension is discussed in relation to enzyme movement in Chapter 7.

Several dyes were incorporated in diets with or without 0.5% tannic acid, these and diets with varying concentrations of tannic acid were fed to fasted adult male cockroaches (Chapter 3). The results gave an indication of the rate of passage of ingesta through the midgut and also demonstrate the possible retention of dyes in the caeca for considerable periods. Cockroaches feeding on diets containing tannin appeared to hold the food longer in the gut, especially by the third day after first feeding. This was also reflected in lower ECD (efficiency of conversion of digested food) values and mean faecal weights after five days of exposure to diets containing 0.1% or 0.5% tannic acid.

Various dyes were also injected into the abdomen of the adult male and female cockroaches under various conditions (Chapter 3). All dyes were commonly absorbed by the Malpighian tubules and then secreted into the lumen of the ileum where they were adsorbed onto the PM. On a few occasions they were seen in the caeca and even in the crop and such observations, although not in themselves indicative of any specific process, would certainly be consistent with a countercurrent system as suggested by Dow (1986).

The rate and type of production of the PM might influence its permeability and the interaction of tannin with it. By injecting labelled chitin and protein precursors, [C14]-N-acetyl-D-glucosamine and [H3]-cystine dihydrochloride respectively, into fasted cockroaches and then allowing them to feed, it was found that the labels were first incorporated into anterior sections of PM (Chapter 4). These and other results suggested that the PM production was of Type II (Chapters 2 and 3) and in response to stimuli not exclusive to feeding.

Electron microscopy (Chapter 5) showed changes in the ectoperitrophic surface layer along the length of the PM which could conceivably cause differences in permeability of the PM along its length. Ingested tannic acid also affected the ectoperitrophic surface layer, causing it to become more open in certain sections and this was thought likely to affect

permeability. Peters and Kalnins (1985) observed immobilised aminopeptidases on the ectoperitrophic surface of the PM in P. americana, thus any changes in its structure may also affect protein digestion and assimilation.

PM sections from female adult cockroaches that had access to a diet with or without 0.5% tannic acid were tied onto a needle to form a blind sac. These sacs were perfused with water or aqueous solutions of various dyes, and bathed in either water or a saline solution (Chapter 6). Passive transport of water was apparently independent of the original position of the PM in the gut, nor did such transport vary greatly when cockroaches were fed diets containing tannin, or when PMs were taken from females after 2 hours or three days access to food. There was, however, a significant affect of the dyes themselves on the hydraulic conductivity (passive water transport). Small, charged dyes (<1300 Dalton) increased hydraulic conductivity, presumably due to charge effects, since saline bathing solutions also increased the hydraulic conductivity. The perfusion of PM sacs with an aqueous solution of an albumin-dye complex (ca. 70,000 Dalton) in a bathing solution of water caused the hydraulic conductivity to fall to neglible levels, also leading to little movement of the protein-dye complex through the membrane. When the bathing water was changed to saline, however, the hydraulic conductivity increased significantly with an associated increase in movement of the protein-dye complex across the PM.

The above effects caused by saline were also reversible, suggesting that if the insect could control ionic composition or concentration in the midgut lumen, there may be a regulation of enzyme movement across the PM.

The possible involvment of the PM in digestion and assimilation of nutrients, as well as the effects of tannic acid on the PM and their possible consequences on digestion and assimilation, are discussed in Chapter 7.

CHAPTER 2 : General methods and initial observations on the foramtion of the PM.

2.1 Culture Method for P. americana.

P . americana were obtained initially from a culture kept by the Waite Institute Insectary and were raised and bred in several large plastic tubs (80 cm x 40 cm x 40 cm) with unrestricted access to mouse food (Milling Industries, Mile End) and water. Shelters were provided by rolls of corrugated cardboard. Vermiculite (grade 2) covered the bottom of the container. Temperatures were maintained at 27-30 ^{o}C and lighting was natural.

Unless otherwise stated, the cockroaches used in experiments were taken from general culture and placed in well ventilated containers (150 ml plastic vials with wire mesh vents in sides and lids) without food but with water ad lib for five days. They were kept at 27 $\pm$ 2 ^{o} C in "reverse lighting" with a ten hour scotophase beginning 1300 hrs. Unless otherwise stated, the cockroaches were then fed for three days on a standard protein-free artificial diet (section 2.5). Most dissections were begun at the commencement of the scotophase, i.e. to coincide with the assumed active period.

2.2 **Dissection of cockroaches.**

The gut, from crop to rectum, was dissected from cold-anaesthetized insects on a wax dissecting dish under saline. Except when stated otherwise, the saline used for dissections was that of Miles and Slowiak (1976) : 7.5 g NaCl, 1.2 g KCl, 1.0 g $CaCl_2$ (anhydrous), 0.4 g $MgCl_2.6H_2O$, 0.6 g $NaHCO_3$, 0.4 g CH_3OONa (anhydrous), and 0.55 g KH_2PO_4 in 1 l, all reagents of analytical or laboratory grades; if tissues needed to be kept alive for long periods, 11.0 g of glucose was also added. The gut was uncoiled and pinned to present the midgut as a straight tube from which fatbody and tracheae were stripped away. Since the midgut had a marked hydrostatic pressure relative to the external saline bath, it was necessary to excise a few caeca to equilibriate the pressure of the midgut lumen with that of the bathing solution. The midgut epithelium was stripped away from the PM with fine forceps starting near the Malpighian tubules and moving towards the caeca in much the same manner as decribed by Lee (1968). The PM is thereby left as a slightly curved cylinder attached to the oesophageal invagination. For each dissection routine notes were taken of crop contents, caecal contents, contents of each third of PM, contents of hindgut, and structure of each third of PM (see 2.2.5). The location and a subjective assessment of relative concentration of brown fluid (see section 2.2.5) and/or any dye if used were also recorded.

2.3 Subjective assessment of structure of the PM and relative concentration of brown fluid.

PMs were arbitrarily divided into thirds thus giving sections from the anterior, middle, and posterior region of the midgut. PM sections were therefore referred to as anterior PM (antPM), middle PM (midPM), and posterior PM (postPM) respectively. Each section was subjectively assessed for its capacity for plastic deformation and its age. In practice, the former was determined on whether the PM section appeared "fluid" or "brittle"; antPM sections often seemed "fluid", i.e. there was an apparent "streaming" (i.e. plastic deformation) of PM when pulled away from the oesophageal invagination, while something approaching brittleness was commonly seen in midPM and postPM sections, i.e. the PM would fracture relatively easily and uniformly when torn or cut. Fluid PM from the antPM was usually coloured brown by the contents of the caeca (see below) although the colour could be washed out in sections separated from the gut. Colourless, fluid PM obtained in this way appeared very fine, almost completely transparent and for this reason was particularly difficult to handle, e.g. in permeability experiments (Chapter 6).

Where not associated with brown caecal fluid, the PM was typically more substantial. Its age was gauged on how translucent or coloured it was due to occlusion of the

endoperitrophic surface with food debris; i.e. transparent PMs were classified as "new" since these were normally found in cockroaches that had been feeding within three days of the dissection, while cloudy and coloured, brittle PMs were considered "old" because they were typical of cockroaches that had been deprived of food and were unlikely to have recently produced PM to any great extent.

As mentioned above, a soluble brown pigment was found in caecal lumina and the anterior region of the midgut lumen. Lee (1968) also notes the close association of "brown material" with PM from near the caeca and assumes the brown material is secreted by the caeca. The distribution of this soluble brown pigment was therefore assumed to reflect, in part, distribution of solutes in the midgut (see also Dow, 1981a).

2.4 **Observations on formation of the PM.**

Initially, cockroaches (adult and juvenile) from general culture (i.e. fed ad lib) were dissected to obtain an overall impression of the PM in untreated individuals. Also, both cockroaches and adult Lucilia cuprina (see below) were dissected (as in 2.2) within an hour of ecdysis and notes made of the appearance of the PM.

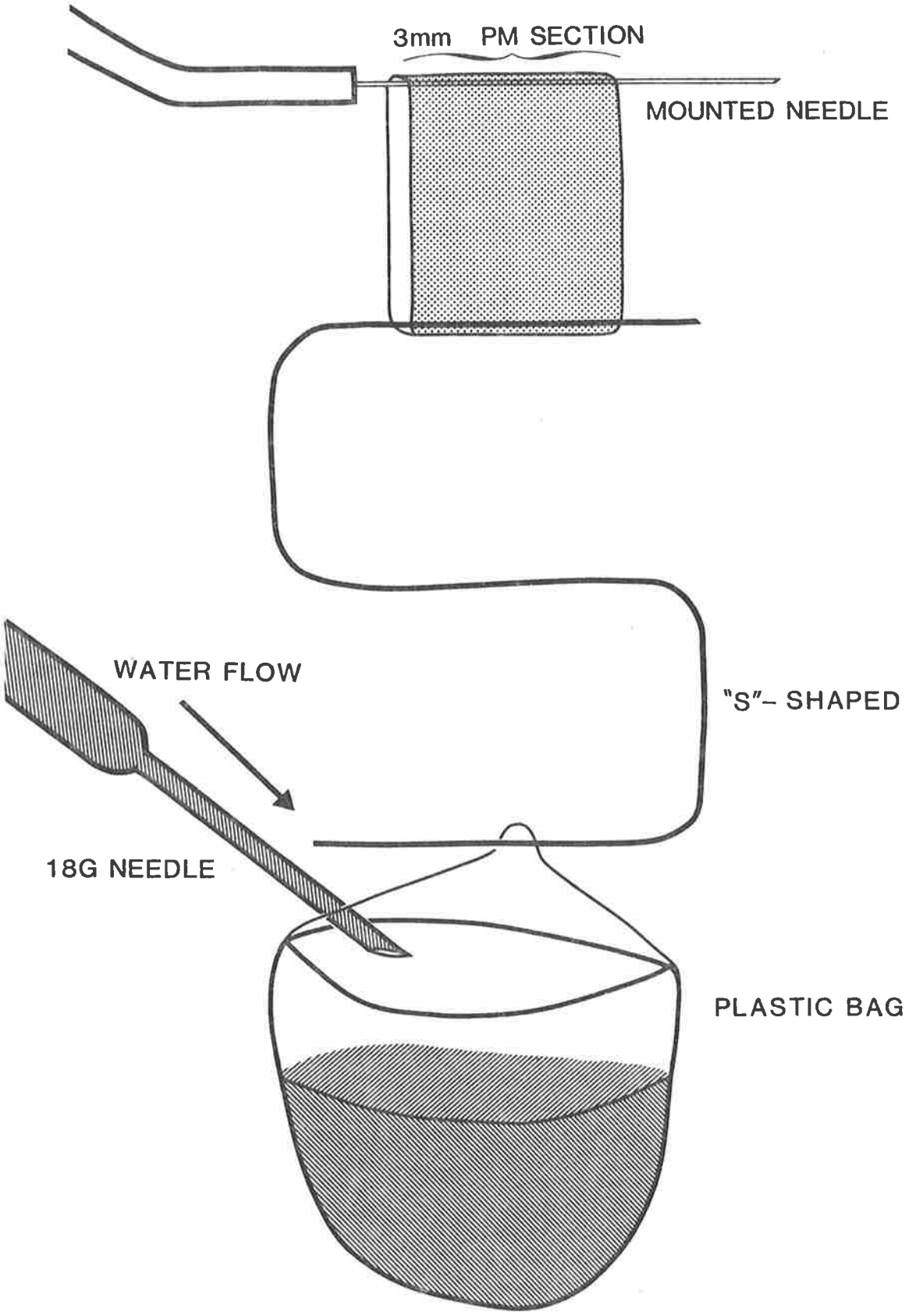
2.4.1 Initial observations on the production and filling of the PM.

The first impression of the PM dissected from cockroaches that had recently fed on mouse food and vermiculite was that of a very translucent and hyaline cylinder of more or less uniform diameter with ingesta compacted into it for most of its length.

In some cockroaches that had been deprived of food for five days and then fed 0.5% tannic acid incorporated in the standard diet (diet 5b, see below) the antPM gave the appearance of a series of bulges, each formed successively on the bulbous tip of the oesophageal invagination, which had somehow acted as a template (see Figure 2.2). Such "sinuate" PM in the anterior region of the midgut, where it was not coloured by caecal fluid, was more transparent and more substantial than PM of more uniform diameter taken from the same gut region of other cockroaches. On the ectoperitrophic side of the sinuate membrane, a further, very fine, smooth layer overlay the main bulk of layers which formed the bulges. In dissections, this fine, superficial layer never appeared intersposed between the caecal lumina and the rest of the PM; either it readily broke away from a source anterior to the opening of the caeca, or more likely, it arose posterior to, or at the base of the caeca. The fine layer may be analogous to the amorphous granular layer seen with Scanning Electron Microscopy in Chapter 5.

Figure 2.1

Apparatus for measuring extension and loading capacity of PM sections.


3mm PM SECTION
MOUNTED NEEDLE
WATER FLOW
"S"- SHAPED
18G NEEDLE
PLASTIC BAG

When PM sections showing sinuate bulges (i.e. from cockroaches fed tannic acid) were tied on to a needle to form a blind sac and were then perfused with water (see Chapter 6), they would expand at pressures of about 200-300 mm H20 to about twice their initial length. The hydraulic conductivity of these sections before expansion seemed in some cases very low but once expanded, the hydraulic conductivity would become comparable to that of "normal" sections of PM (i.e. from cockroaches fed other diets). Thus, the sections showing bulges were not entirely "brittle" nor were they entirely "fluid". Such folding and transition to a more substantial, transparent form in the region close to the oesophageal invagination would seem consistent with production of PM faster than its passage backwards towards the ileum. These folded PMs always seemed to lack enclosed ingesta, implying perhaps that crop pressure may facilitate movement of PM backwards. The concept that PM movement is affected both by movement of food and by the influence of crop pressure is supported by the observation that PMs from the posterior region of the midgut of cockroaches that were deprived of food for five days were also folded. Further implications of these observations for the type of production that may occur in the roach will be discussed below (2.4.2).

There was an area posterior to the oesophageal invagination where solids from the crop spilled out into the PM lumen after passage through the oesophageal invagination. Pressure from the crop probably forces food backwards to the

main body of compacted ingesta *in vivo*. The PM overlies the oesophageal invagination and immediately behind the tip of the invagination is a length of about 2-3 mm of PM where the PM appeared to be less substantial than further on and slightly fluid in character. Normally this fluid PM was found in association with liquid containing the natural brown pigment (see above, 2.3). In cockroaches from general culture that did not appear to be actively feeding (and in which the crop was not usually very full but there was some food throughout the gut), the PM anterior to the oesophageal invagination sometimes appeared to have a peculiar "crown effect" such as also described by Lee (1968). The points of the crown corresponded to the origins of the caeca which had presumably influenced the shape of the PM during its formation in these particular insects.

The PM normally occupied the length of the midgut and ileum and appeared to be broken down in most instances in the anterior colon by either muscular contractions of the hindgut, or the oxyurid nematodes that were normally present in the cultures.

2.4.2 **PM from teneral cockroaches.**

Observations on PMs from recently moulted cockroaches (and before they had ingested exuviae) showed PMs that were darkly pigmented. Unlike the colouration of brown fluid of PM

in the anterior midgut, the pigmentation of PMs associated with ecdysis would not wash out. Along the length of such PMs there were distinct creases in the ectoperitrophic surface and sometimes a gradation of colour from brown to black, anterior-posteriad. Also, in the posterior region of the midgut there was marked folding and compaction of the PM, so that a PM up to two lengths of the midgut could be found in this region. Hindgut contents, including the oxyurid nematodes normally present, were ensheathed in the old cuticular intima. Adult Lucilia cuprina (reared as in section 2.7) also had a darkly pigmented PM at the time of ecdysis although it did not appear to be folded.

The appearance of a dark brown PM at ecdysis contrasts sharply with the unpigmented teneral cockroach. Since the PM at this stage was always empty, it is possibly secreted sometime before or during the moult. Synthesis of PM at this time would be consistent with observations in vitro on cultures of dipteran cardia cells (a band of PM producing cells in the anterior midgut of some flies) where 20-hydroxy-ecdysone stimulated the production of PM (Becker, 1978). One of the PM's function at this time could be the absorption of, and protection of the midgut epithelium from moulting fluid. Cornell and Pan (1983) showed the entrance of moulting fluid after ecdysis into the midgut of a lepidopteran (Manduca sexta). They suggested that this was the main method of absorption of moulting fluid in insects since it had also been

found in elaterid larvae (Zacharuk, 1973 cited by Cornell and Pan). The colouration of the PM in the roach could result from the same thing and, when the insect moults, the PM could serve to absorb moulting fluid components that might adversely affect the midgut epithelium. Although precisely what causes the darkening appears problematical since it occurs at a time when the roach itself is "lilly" white.

The finding that the pigmented PM at the time of ecdysis is continuous but highly compacted in the posterior region of the midgut, and the formation in cockroaches fed tannic acid of bulges of PM apparently moulded by the tip of the oesophageal invagination and then passed backwards is hard to reconcile with the suggested Type I production of PM in P. americana (see Chapter 4). It would appear instead that the PM is produced by cells of the anterior midgut, and is then pushed backwards; at ecdysis, the constriction at the midgut and ileum junction apparently prevents the PM from passing back into the ileum so that continued secretion caused it to back up into folds.

2.5 **Assessment of suitability of various artificial diets.**

Dissection of cockroaches from general culture showed that they ingested substantial amounts of inert substances (i.e. vermiculite) in their diet which is assumed to reflect their non-discrimatory feeding (in Cornwell, 1968). With this in view, attention was given to incorporation in artificial

diets of non-nutritional substances that would be passed out with the faeces (c.f. Bignell, 1978, who used cellulose as a dietary diluent).

Mouse pellets were considered undesirable not only because of their unknown composition but also because they contained fibre that could not be removed from dissected PMs because of its irregular shape and tendency to catch on the lumen surface. Therefore, several artificial diets (see below) without protein were qualitatively assessed for their palatibility, ease of incorporating material, and their ability to be washed off the PM (i.e. "washability"). This last quality was particularly necessary for work on the permeability of the PM (Chapter 6). That the diet should be protein free was essential for subsequent work with incorporation of tannic acid and gallic acid in the diet (Chapters 3, 5 and 6). Since it would be used for only a few days, the lack of protein or other nitrogen sources was not considered to be particularly detrimental to the nutrition of the roach, especially since adults only were used.

Artificial diets consisted of a gel of starch (Whatman AR grade) containing sucrose as a phagostimulant (LR grade) and added components; the weight ratios were 40 starch : 20 sugar : 20, 40 or 80 of insoluble constituent as in Table 2.1. Ingredients were mixed in the appropiate ratio in a glass beaker with 90 ml of water added to every 20 g of starch used.

TABLE 2.1 : Artificial diets used in the assessment for suitability for feeding to *P. americana*.

DIET No.	INGREDIENTS*			RATIO
1a	Starch	Sucrose	Glass Beads	40:20:40
1b				40:20:80
2a	Starch	Sucrose	Carborundum	40:20:20
2b				40:20:40
3a	Starch	Sucrose	$CaCO_3$	40:20:20
3b				40:20:40
4	Starch	Sucrose	Filter Paper	40:20:#
5a	Starch	Sucrose	Silica Gel	40:20:20
5b				40:20:40

* See text for details about making into a dried cake.
\# Mixture was coated onto Whatman No.1 Filter paper discs.

The mixture, continuously stirred, was heated, turning first viscous and then more fluid, and then poured into 9 cm glass petri dishes. The mixtures were allowed to cool forming gels which were then dried in an oven at 200 ^{o}C. In one feeding trial, paper was coated with the starch/sugar solution was tried.

Each diet, as described in Table 2.1, was given to a group of 4 adult females. Groups were allowed to feed on the diet for three days as described in section 2.1 and then placed at 4 ^{o}C for an hour prior to dissection. PMs were removed as described in section 2.2 and then qualitatively assessed for palatability, washability, and overall ease of preparation.

Washability was assessed for either 4 mm or 8 mm sections of translucent PM as follows: the lumen of the PM was pierced with a fine needle and then washed with a flow of water from a pipette; the PM was then cut in half and the lumen surface inspected for the area still occluded by the diet.

Palatability was assessed from the amount of ingesta in the crop, midgut and hindgut.

Results (Table 2.2) indicated that diet 5b (Table 2.1) consisting of starch, sucrose and silica in the ratio of 40:20:40 was the best overall in that it had good washability and maintained PM integrity and was reasonably palatable. In

Table 2.2 : Assessment of suitability of various insoluble components of diets in for feeding to *P. americana*.

DIET	No.	RATIO*	WASHABILITY 4mm PM section**	WASHABILITY 8mm PM section**	INTEGRITY of PM	PALATABILITY of DIET
Glass	1a	40:20:40	+++	++	++	+++
	1b	40:20:80	+++	+	++	++
Carborundum	2	40:20:20	+++	+++	++	++
$CaCO_3$	3a	40:20:20	++	+	+++	++
	3b	40:20:40	++	+	+++	+++
Filter paper	4	40:20:#	++	-	+++	+++
Silica Gel	5a	40:20:20	+++	+++	+++	++
	5b	40:20:40	+++	+++	+++	++

* Ratio of Starch/ Sucrose/ Diet ingredient (See Table 2.1).
** PM section from the middle or posterior region of the midgut.
\# Starch/ Sugar mix was coated on filter paper.
+++, ++, + = degree of positive result.
\- = negative result.

subsequent experiments (see below and Chapters 3, 4, and 5) therefore, diet 5b in Table 2.1 was used together with other additives as required; it will be referred to as the "standard diet".

Some other diets showed adverse affects not shown in the table. Glass beads in the diet proved too difficult to handle and a tendency to separate out from the other ingredients. When cockroaches were fed diet containing carborundum, wax droplets were found in the crop. Since no lipids were present in the diets and the foregut is not known to have a secretory nature, it must be assumed that the droplets were derived from abrasion of cuticle of some part of the mouthparts or foregut. Nevertheless, the PM conveniently packaged the carborundum, passing it through to the hindgut, thus providing powerful evidence in support of the concept that the PM protects the midgut from abrasion by solid particles (Wigglesworth, 1972 c.f. Richards and Richards, 1977). Ingested calcium carbonate had marked effects on the appearance of the alimentary system: no brown pigment or any associated fluid was seen in the anterior midgut or caeca; the PM however, seemed largely unaffected and still appeared translucent.

2.6 Measurement of extension and breaking load in PM sections.

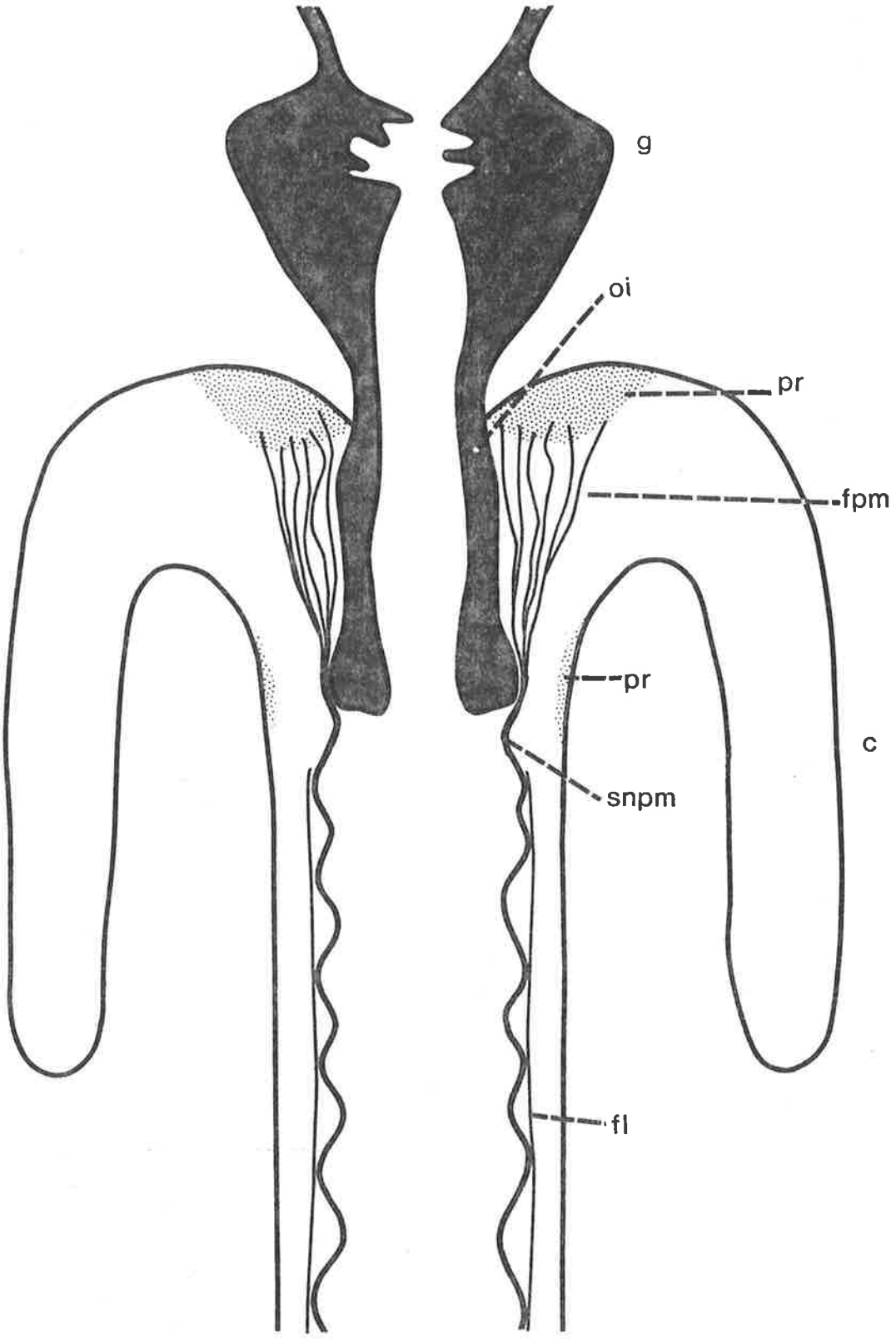
The PM was dissected from adult male cockroaches (as in 2.2) that had been feeding on the standard diet (described in 2.5) for three days and 3mm sections were taken from various regions of the midgut (i.e. the antPM, midPM and postPM as in 2.3). The open-ended cylinders of PM thus formed were slipped onto a needle that was mounted in a plastic holder (Figure 6.2). A pin shaped as an "S" was slipped through the PM section and allowed to hang. Through the bottom bend of the S-shaped pin was hung a small plastic bag with about a 2 ml capacity. Water was added to the plastic bag with a Pharmacia P-3 peristaltic pump at a rate of 1 g/min. Extension of the PM was measured with a gradiculus in a binocular microscope. The time taken until breaking was recorded and the plastic bag and contents then weighed. Sections were kept moist with distilled water added from a pipette.

Percentage extension was calculated from the observed extension when water was added to the plastic bag as a percentage of the original length measured without any load. The breaking load was that at the time of breakage, measured as indicated above.

PM sections from the anterior, middle and posterior region of the midgut (antPM, midPM and postPM respectively)

Figure 2.2

Model of formation of sinuate PM in some cockroaches fed tanic acid for three days. (c) caecum, (fl) fine layer, (fpm) fluid PM, (g) gizzard, (oi) oesophageal invagination, (pr) PM precursor, (snpm) sinuate PM. See Text for details.


g
oi
pr
fpm
pr
c
snpm
fl

showed little difference for either mean extension or mean breaking load; approximately 15-17% and 1.20-1.24 g respectively (Table 2.3).

2.7 **Other animals used in this study.**

Other insect species used for occasional comparisons were Panesthia cribrata (Saussure) (Blattodea : Blaberidae), Chorticetes terminifera (Walker) (Orthoptera : Acrididae) and Lucilia cuprina (Wied.) (Diptera: Calliphoridae).

Pa. cribrata was collected from Armidale (NSW) and some were also provided by Dr H. Rose, University of Sydney. These cockroaches feed on wood and often live in rotting logs or the ground below them. Under general culture, they were kept in the same conditions as P. americana (see 2.1) except the tub in which they were kept was provided with 10-20 cm of soil and pine wood chips and shavings which were kept moist with a water bottle and cotton wick.

Ch. terminifera eggs were provided by Dr P. Gregg, Unversity of NSW and incubated at 32 ^{o}C. They were reared at 38 ^{o}C in large cages consisting of a frame (50 cm x 50 cm x 40 cm) covered with fly wire. It had a mesh screen above a sloping floor which allowed frass to drop though and be collected below. In the cage was a 60W lamp with wire mesh near it so that the grasshoppers could bask. The grasshoppers were fed

tender shoots of grasses in season which were changed daily while cages were cleaned of frass and wiped with 0.1% sodium hypochlorite.

L . cuprina larvae obtained from Dr C. Cooper, University of Adelaide were reared on liver lying on vermiculite in a small plastic tub (1 cm x 2 cm x 1 cm) with wire gauze vents. Pupae were transferred to a plastic tub (30cm x 20cm x 20cm) which was well vented. After emergence, the flies were given access to milk powder, sugar and water. A week after emergence, liver was provided as an larvipositional site for the female. Larvae were transferred five days later to fresh liver. All stages of the life cycle were kept maintained at a temperature of 30-32 ^{o}C.

Table 2.3 : Means of %Extension and breaking load of PM sections from different regions of the midgut in adult male roaches. Number of replicates (n) and standard error (SE) in brackets.

PM SECTION	%EXTENSION* (n, SE)	BREAKING LOAD** (n, SE)
antPM	16.7 (11, 1.9)	1.26 (11, 0.21)
midPM	15.6 (16, 1.6)	1.20 (16, 0.16)
postPM	16.9 (24, 1.6)	1.24 (24, 0.12)

* %Extension was calculated from the observed extension when water was added to the plastic bag as a percentage of the original length measured without any load.

** The breaking load of the PM that at the time of breaking.

CHAPTER 3 : Feeding experiments with diets containing different concentrations of tannic acid, and movement of solids and solutes through the gut.

3.1 Introduction.

Dow (1986) provides a review of the general function of the midgut in insects and emphasises that absorption capacities in some insects could be maximised by a countercurrent system of solute movements in the ectoperitrophic space (as described by Dow 1981a). For cockroaches however, there is nothing conclusive to support this model. Although not intending to test Dow's model rigorously, it was hoped to provide some insight on the movement of dyes in relation to the movement of solids (carried by the PM to the hindgut).

Bernays (1980) has suggested that from an evolutionary stand point, phytophagous insects had become adapted to cope with condensed tannins at an early stage in evolution, but among the Acridoidea the ability to cope with hydrolysable tannins (e.g. tannic acid) was secondarily lost in graminivorous groups that radiated with the Gramineae (a plant family deficient in tannins). Tannins are normally considered to have antidigestive or antifeedant properties, these effects being quantitative. Bernays (1981a), in her appraisal of the relation between insects and plant tannins found there was

evidence for antifeedant properties of both condensed and hydrolysable tannins on insects that do not normally encounter tannin in their diet. This was at low concentrations in some (e.g. lepidopteran species) but at much higher levels for others (e.g. graminivorous grasshoppers). Hydrolysable tannins also had definite phagostimulatory effects for some species, but true toxic effects at high enough concentrations. Antidigestive effects have so far been shown only *in vitro*, however.

In view of the ideas and results indicated above, it was of interest to determine whether tannic acid would show a toxic effect when ingested by the cockroach, a generalist feeder. The importance of the PM as a barrier to tannic acid has been reported in *Schistocerca gregaria* (Bernays and Chamberlain, 1980) and its toxic effect on the midgut epithelium once it had penetrated the PM in graminivorous species of Acridoidea (Bernays *et al.*, 1980). One of the objects of the present research was, therefore, to investigate the effect of ingestion of tannic acid on assimilation in the cockroach, especially in relation to its effects on the structure and permeability of the PM. In order to do this, it was necessary to determine the extent to which cockroaches would consume diets containing tannic acid.

This chapter provides information concerning the feeding patterns and weight gain in cockroaches when feeding on diets

with or without tannic acid, and also the movement of solids and solutes through the gut. Three main approaches were used: (i) Various dyes were incorporated into a standard diet with or without tannic acid to observe movement of both solids and solutes through the gut. (ii) Various concentrations of tannic acid were added to the diet without dye to test the effect of ingested tannin on digestion. (iii) To investigate the possibility of a countercurrent flow, dyes were injected into the abdomen of adult female and male cockroaches to detect any water flow to the caeca from a posterior source (e.g., Malpighian tubules).

3.2 Materials and Methods.

3.2.1 Incorporation of dyes and tannic acid in an artificial diet and determination of dye and food movements and effects of tannic acid on ingestion and digestion:

To understand movement of food in both its fluid and solid phase in relation to the structure of the PM, groups of four adult male cockroaches (per treatment and at each dissection time) were fed artificial diet with various dyes incorporated. Dyes were added as 90 mls of 0.5 mM aqueous dye solutions. In the first feeding trial for determination of the rate of passage of solids and solutes, the dyes used were Azure B (Gurr) 5-aminoacridine hydrochloride (Laboratory Reagent),

Orange G (Chroma, Schmid and Co.), and Fast green FCF (Pharmaceutical Laboratories). The second feeding trial was a comparison of the rate of movement of diets with or without 0.5% tannic acid, and the dyes added to the diet were Fluoroscein (Gurr), Azure B (Gurr), Saffranin O (Gurr), and Methylene blue (Merck). Cockroaches were prepared for feeding trials as in Chapter 2 and then fed as described below. A feeding trial day was defined as the 24 hour period commencing at the beginning of the scotophase; this would reflect more closely the activity cycle of the roach since onset of feeding occurs in male cockroaches within the first hour of scotophase (Sutherland, 1982).

In the first feeding trial, groups of five adult cockroaches were fed diet with dye as summarised in Table 3.1.a. Diet 1 with Azure B was fed to the cockroaches at the commencement of day 1; twenty two hours latter, i.e. two hours before the scotophase beginning day 2, diet 1 was replaced with diet 2 containing 5-aminoacridine; diet 3 with Orange G replaced diet 2 at the beginning of day 2; diet 4 with Fast green FCF replaced diet 3 one hour prior to the next scotophase, i.e. at the commencement of day 3.

Cockroaches were dissected as in 2.2 but fluid samples of crop and caeca were taken with a two µl microcap before the gut was immersed in saline. Notes were also made of location of diets and gradation of dye intensity of the compacted ingesta

Table 3.1 : Dyes incorporated into an artificial diet, and time schedule for feeding of cockroaches with diet and dissection of cockroaches.

3.1 (a) : Feeding Trial 1:

Diet No.	Diet	Day and Hour Fed	Day and Hour Dissected
1	Azure B	Day 1, Hour 0	Day 1, Hour 0,1,2,3,4 and 22
2	5-aminoacridine	Day 1, Hour 22	Day 1, Hour 23 and 24
3	Orange G	Day 2, Hour 0	Day 2, Hour 1,2,3,4, and 23
4	Fast green FCF	Day 2, Hour 23	Day 3, Hour 0,1,2,3,4,23 and 24

3.1 (b) : Feeding Trial 2:

Diet No.	Diet	Day and Hour Fed	Day and Hour Dissected
1	Fluoroscein	Day 1, Hour 0	Day 1, Hour 2
2	Methylene blue	Day 1, Hour 2	Day 2, Hour 0
3	Saffranin O	Day 2, Hour 0	Day 3, Hour 0
4	Azure B	Day 3, Hour 0	Day 4, Hour 0

along the length of the midgut and ileum. Dissections of four adult male cockroaches were made hourly in a six hour period starting two hours prior to the commencement of each scotophase except for day 1 when no dissection was made during the two hours before cockroaches had access to the first diet. Fluids and any semi-fluid mixes were spotted on filter paper (Whatman, Grade 1) and then ran in 70% ethanol (ethanol/ water) to separate the dyes.

To check the effect of tannic acid on ingestion of P. americana, 24 adult male cockroaches were prepared for a similar feeding trial as above (feeding trial 2), but with different dyes and either with or without 0.5% tannic acid. Table 3.1.b summarises the dyes used and the times at which the diets were fed to the cockroaches. Diet 1 with Fluoroscein was fed to the cockroaches at the beginning of day 1 and was replaced with diet 2 containing Methylene blue two hours later. Diets 3 and 4 were offered at the beginning of days 2 and 3 respectively, replacing the previous diets as before. Dissections of four cockroaches per treatment were made at the time of the exchange of diets.

In a third feeding trial, tannic acid at concentrations (with respect to final dry weight) of 0%, 0.05%, 0.1%, and 0.5% were incorporated into the standard diet (5b in Table 2.1). Five adult male cockroaches were used for each test concentration and prepared as above. Before the cockroaches

were fed, both insect and food were weighed. They were dissected after five days and dry weight of food and faecal pellets, and final body weight were measured. From these, Consumption (C), Approximate Digestibility (AD), and efficiency of food conversion (ECD), were determined (Waldbauer, 1968).

3.2.2 Injection of dyes into the abdomen of fasted and feeding cockroaches under various conditions.

Cockroaches were deprived of food as in Chapter 2 and then either (i) injected and dissected, (ii) injected and left to feed for various times, or (iii) fed mouse food (see 2.2.3) for five days, injected, and dissected at various times thereafter. Injections and dissections were performed in scotophase and photophase for comparison of dye movement in different phases of the feeding cycle. Both adult male and female cockroaches were used. 5 or 10 µl injections of 0.5% Azure B, Saffranin O, or Trypan blue were given through the side of the intersegmental membrane of the seventh abdominal sclerite. Cockroaches were dissected as in 2.2.3 and the location of dye noted.

3.3 Results.

3.3.1 Passage of dyes incorporated in an artificial diet through the gut of *P. americana*.

Results from feeding trial 1 are summarised in Figure 3.1. and presented below in a semi-tabulated form. The four dyes used in diets 1, 2, 3, and 4 respectively as listed in Table 3.1 will hereafter be referred to as dyes 1, 2, 3, and 4. Since these dyes were soluble in water, they were used as markers for solute movement within the gut and therefore may not necessarily have indicated the distribution of the insoluble components (or other solids) with which they were ingested. For the most part, there were solid ingesta throughout the whole length of the gut except for (i) the first two to three hours of day 1 when the ingesta were in the process of passing down the previously empty midgut and (ii) again when the crop and midgut cleared prior to a second feeding peak on day 3 (within the first four hours of day 3).

DAY 1: Diet 1 offered to cockroaches just before beginning scotophase.

Hour 0 (i.e. commencement of scotophase): Before ingestion commenced, dark brown fluid could be seen in the anterior midgut and about 75% of the PM appeared old (as defined in 2.3).

Hour +1 (i.e. one hour into scotophase): Oesophagus and crop were filled. (Figure 3.1.a).

Hour +3: Ingesta were present in crop, midgut and ileum (Figure 3.1.b). Dye 1 was found in the caeca and ectoperitrophic space.

Hour +4: Ingesta were present in crop, midgut, ileum

and anterior colon. (Figure 3.1.c). There was a noticable decrease in intensity of dye associated with ingested diet in the crop.

Hour +1 to +4: The distribution of brown pigment along the length of the midgut appeared to be associated with seemingly increased PM fluidity (as defined in section 2.3) as the old PM was progressively replaced with more fluid new PM. The intensity (i.e. concentration) of the brown pigment appeared to decrease over time (Figure 3.1.a - 3.1.d).

DAY 2: Diet 2 had been offered to cockroaches two hours before beginning of scotophase on day 2; diet 3 was offered at the start of day 2:

Hour 0 (before feeding on diet 3 had begun): (i) Diet 2 was found in the oesophagous and mixed with diet 1 in the crop (Figure 3.1.e). (ii) Diet 1 was found by itself throughout the gut from crop to colon. (iii) Dye 1 appeared to have stained the midgut epithelium along its whole length (Figure 3.1.e), although there was a noticeable decrease in the intensity of dye in the PM contents in the posterior region of the midgut. (iv) The brown liquid at this time and for the two hours into day 2 was restricted to the caeca where its intensity was markedly less than during the first 4 hours after feeding on day 1. Its usefulness as a marker thereafter was diminished due to masking by the ingested dyes.

Hour +1: Only the anterior midgut appeared to be stained

with dye 1.

Hour +2: Diet 3 found in the oesophagus.

Hour +4: (i) Diet 3 found in the crop mixed with a trace of dye 2 (Figure 3.1.f). (ii) Dyes 1,2 and 3 were found mixed in the ingesta halfway down the midgut in some insects. (iii) Mainly dye 3 and some dye 2 were discernible in the caeca and anterior ectoperitrophic region (Figure 3.1.f). (iv) The PM contents in the ileum were nearly colourless. (v) The anterior PM section seemed fairly fluid, the middle and posterior PM sections appearing more substantial and translucent to transparent.

DAY 3: Diet 4 offered to cockroaches one hour before beginning of scotophase:

Hour -2 (i.e. two hours before the commencement of day 3 : (i) Solid ingesta, dye 3 and traces of dye 2 were found in the crop and midgut, and colon (Figure 3.1.g). (ii) Dye 3 and dye 2 were present in the anterior ectoperitophic region and caeca (Figure 3.1.g). (iii) There was a gradation in intensity of dyes anterior-posteriad along the length of the PM with the ingesta colourless in the ileum (Figure 3.1.g). The anterior PM section again seemed fairly fluid.

Hour 1 to 4: (i) The results became more variable, it appeared, however, that the crop and/or the anterior section of PM were emptied of solids. (ii) The contents of the posterior region of the PM and hindgut were solid and contained dye 3 and traces of dye 2. (iii) Dye 3 and dye 2 were still found to be

in the caeca although decreased in intensity (Figure 3.1.h). (iv) During this period, diet 4 appeared to be ingested sometime and dye 4 was then observed in the oesophagus, crop and caeca and throughout the midgut, staining the anterior midgut epithelium (Figure 3.1.i); when present, dye 4 was closely associated with more fluid parts of the PM extending in some insects into the posterior regions of the midgut.

DAY 4:

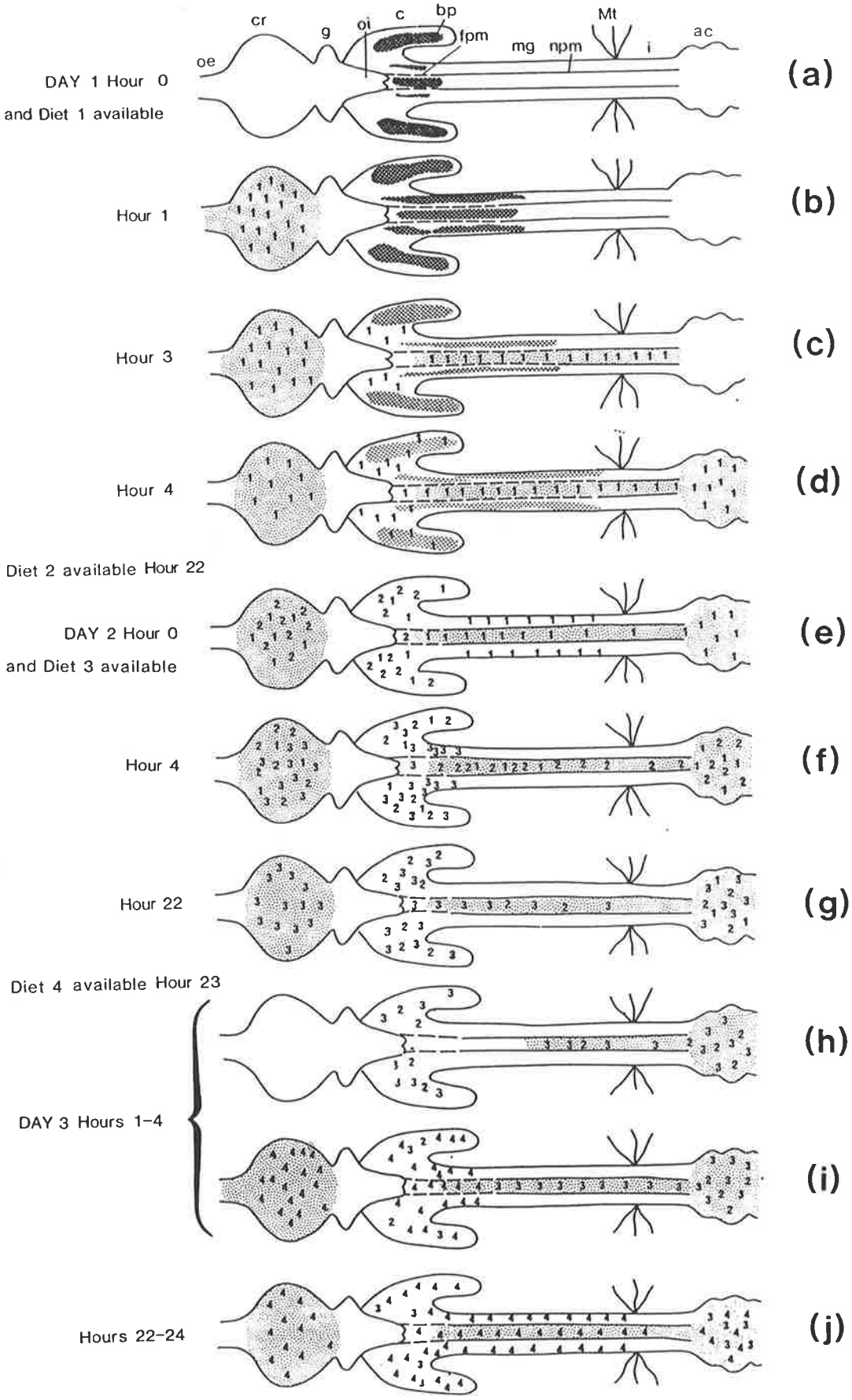
Hour -2 to 0: (i) Traces of dye 3 were found in the colon. (ii) Dye 4 and solids occurred throughout the gut (Figure 3.1.j), staining the posterior midgut. (iii) The ingesta had a less intense colouration in the ileum compared with the ingesta from the midgut. (iv) Dye 4 appeared to be confined to the ectoperitrophic region of the anterior midgut and caeca.

3.3.2 Comparison of movement of diets marked with dyes with or without tannic acid through the gut of *P. americana*.

Details of the methods used in this experiment have been given previously (section 3.2.1). There was no difference between diets with or without 0.5% tannic acid with respect to its distribution and location of dyes and solids throughout the midgut until day 3. The dyes used in the four diets were soluble in water and their use as markers for solute movement

Figure 3.1

(a)-(j) Passage of four diets fed serially to cockroaches over a period of three days. (ac) anterior colon, (bp) brown pigment, (c) caecum, (cr) crop, (fpm) fluid PM, (g) gizzard, (i) ileum, (mg) midgut, (Mt) Malpighian tubules, (npm) non-fluid, transparent PM, (oe) oesophagus, (oi) oesophageal invagination. Numbers in gut represent dye in corresponding diet (see Table 3.2.a); their distribution approximates relative dye concentration. Hatching within crop and PM represents solid ingesta; hatching within caeca and anterior ectoperitrophic space represents brown pigment (bp) and decreases in intensity over time. See text for details.


cr
g
oi
c
bp
fpm
Mt
mg
npm
i
ac
oe
DAY 1 Hour 0
and Diet 1 available
(a)
Hour 1
(b)
Hour 3
(c)
Hour 4
(d)
Diet 2 available Hour 22
DAY 2 Hour 0
and Diet 3 available
(e)
Hour 4
(f)
Hour 22
(g)
Diet 4 available Hour 23
DAY 3 Hours 1-4
(h)
(i)
Hours 22-24
(j)

within the gut was subject to the same constraints as mentioned earlier (in section 3.3.1). For the most part, there were solid ingesta throughout the whole length of the gut except while as it was filled initially within the first 2 hours of day 1.

DAY 1: Diet 1 offered to cockroaches at hour 0; diet 2 offered at hour 2:

Hour 2 (before feeding on diet 2): (i) Solids and dye 1 were found in the crop with brown fluid concentrated in the anterior midgut and caeca. Dye 1 also appeared in the anterior midgut and caeca. (ii) The anterior PM appeared fluid whereas posteriorly the PM appeared older (as defined in 2.3).

DAY 2 : Diet 3 offered at hour 0:

Hour 0 (before feeding on diet 3): (i) Solids and dye 2 from the second diet were found throughout the gut, dye 2 was also found in the caeca. (ii) The anterior PM appeared fluid while posteriorly it appeared substantial and transparent.

DAY 3 : Diet 4 offered at hour 0:

Hour 0 (before feeding on diet 4): (i) Dye 3 from the third diet was found mixed with dye 2 along with insoluble components in the crop and throughout the midgut, although only dye 2 was mixed with the solids in the colon. (ii) The PM appeared to be older posteriorly and the concentration of dyes in the caeca appeared less than in the preceding day.

Day 4: A - Cockroaches fed diets without tannic acid:

Hour 0: Dye 4 from diet 4 (without tannic acid) was found along with solids throughout the gut from crop to colon, and there was some mixing in the colon of dyes 3 and 4.

DAY 4: B - Cockroaches fed diets containing 0.5% tannic acid: Hour 0: (i) Although solids were found throughout the gut from crop to colon, dye 4 was found in the crop only while dye 3 was seen throughout the midgut. There was some mixing of dye 3 with dyes from previous diets in the colon. (ii) The intensity of dye 3 in the caeca was less, and the PM appeared older in cockroaches ingesting diet 4 with tannic acid compared with those ingesting diet 4 without tannic acid.

From the above, it appeared that by day 3 of feeding, addition of 0.5% tannic acid to the diets had slowed movement of food through the gut: without tannic acid, diet 4 was found further along the gut and had reached the colon by the beginning of day 4, whereas with tannic acid, diet 4 was confined to the crop on day 4 and the PM gave the appearance of having aged (presumably as a result of slower secretion) along the length of the midgut.

3.3.3 **Comparisons of diets with different concentrations of tannic acid in feeding trials with *P. americana*.**

Table 3.2 gives results for consumption, per cent weight increases, AD, and ECD and faecal dry weight, for cockroaches

Table 3.2 : Consumption (C), approximate digestibility (AD), efficiency of conversion of digested food (ECD), faecal dry weight and % weight increase for *P. americana* adult males fed artificial diet with different concentrations of tannic acid for five days. Standard Error in brackets.

%TANNIC ACID	C *	AD	ECD	%Weight# increase	faecal dry weight
0	0.82 (0.28)	66.7 (7.6)	60.4 (14.3)	126.4 (5.6)	0.150 (0.019)
0.05%	0.84 (0.22)	72.8 (9.5)	93.2 (48.3)	127.9 (1.5)	0.112 (0.023)
0.10%	0.51 (0.13)	81.2 (3.1)	58.1 (13.8)	122.3 (8.0)	0.072 (0.022)
0.50%	0.78 (0.24)	85.6 (4.2)	41.9 (14.9)	120.8 (4.1)	0.072 (0.015)

* Consumption was measured in g/g initial dry weight.
\# Calculated on a fresh weight basis.

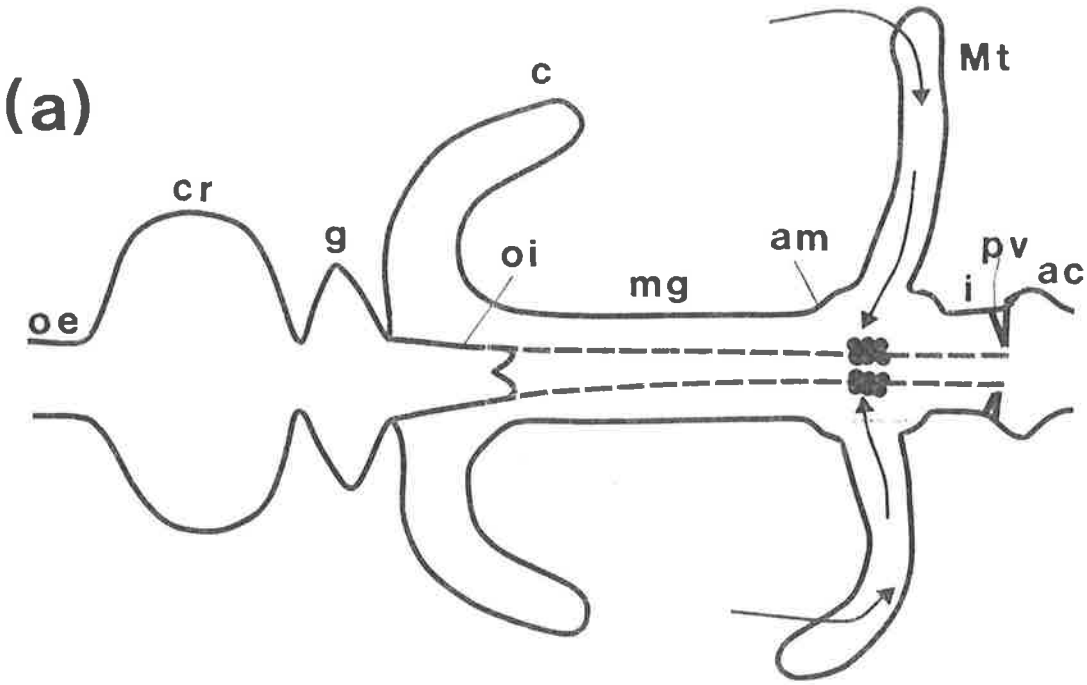
fed diets with 0%, 0.05%, 0.1%, or 0.5% tannic acid for five days. All parameters were similar for treatments and control except for consumption, ECD and faecal dry weight. Consumption for cockroaches fed 0.1% tannic acid was lower than for those fed other concentrations of tannic acid; this value and that for 0.5% tannic acid may be anomalous due to large standard errors, but they generally reflect an apparently lower food intake above 0.05% tannic acid. ECD in cockroaches fed 0.05% tannic acid was much higher than when they were fed other concentrations. Mean faecal dry weights decrease with increasing tannic acid concentration. Also, AD may slightly increase with increasing tannin content but this probably reflected decreased faecal weight.

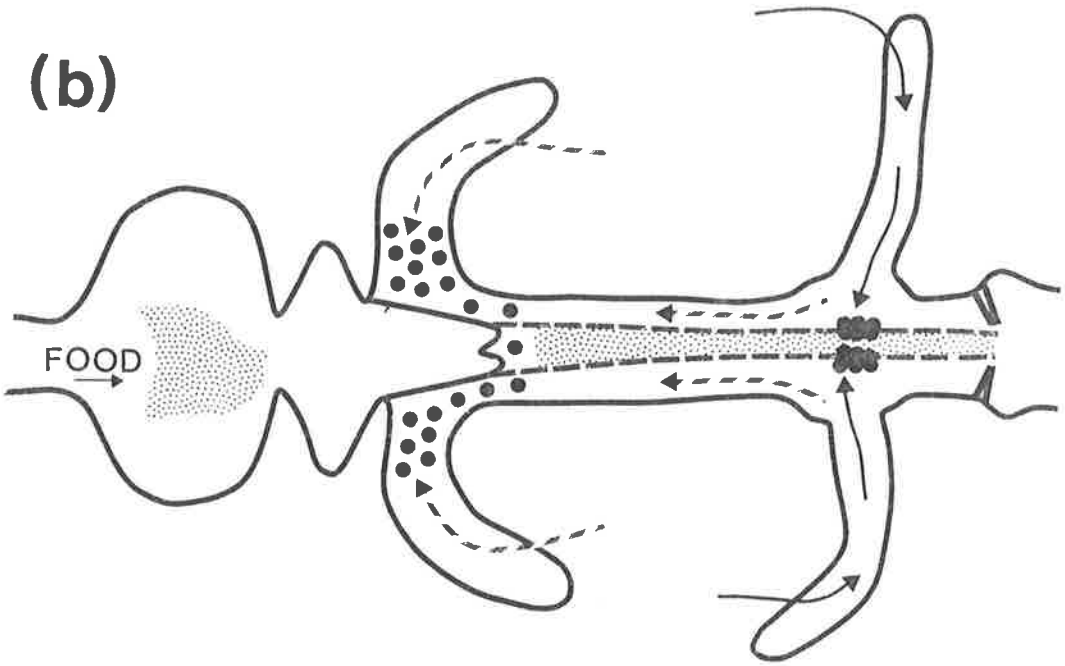
3.3.4 Location of dyes when injected into the abdomen of cockroaches under various conditions.

When adult male cockroaches that had been deprived of food for two days were injected with 10 µl 0.5% Azure B or 0.5% Saffranin O at the commencement of the scotophase or middle of the photophase and left for 20mins, 1hr or 3hrs without access to food, dyes were found adsorbed onto the PM as a band in the ileum anterior to the pyloric valve (the valve at the junction of the ileum and anterior colon), irrespective of time (see Figure 3.2.a). Dyes are presumed to have entered the alimentary tract via the Malpighian tubules since these also had dye in them on most occasions. Since similar results were obtained up

Figure 3.2

Location and possible flows of dyes in the alimentary tract: (a) from cockroaches deprived of food for five days; (b) from cockroaches that had been allowed access to food for some period. (ac) anterior colon, (am) ampullae, (c) caecum, (cr) crop, (g) gizzard, (i) ileum, (mg) midgut, (Mt) Malpighian tubules, (oe) oesophagus, (oi) oesophageal invagination, (pv) pyloric valve. Solid arrows denote probable flows; broken arrows denote other possible flows.


(a)
c
Mt
cr
g
oi
am
pv
ac
mg
i
oe


(b)
FOOD

to 3 hours after injection, they indicate a negligible production of PM in starved cockroaches, except for an initial, small elongation, sufficient to move the stained band of PM a short distance posteriad of entry of the Malpighian tubules (discussed in 3.4.1).

If the cockroaches were allowed access to mouse diet for 1.5 or 2.5 hours into the scotophase before the injection, in those that had a full crop the dyes could be found on the PM in the ileum (as noted above, for fasting cockroaches), and also in the caeca and often in the ectoperitrophic region of the anterior midgut or even in the crop. If the cockroach had not fed, however, then the dyes were again found only adsorbed onto the PM in the ileum, anterior to the pyloric valve. As above, the dye was presumed to have entered via the Malpighian tubules since these again had dye in them on most occasions.

Five female cockroaches that had already had access to mouse diet for five days were injected at the commencement of the scotophase with 10 μl 0.5% Azure B and dissected one hour later after continued access to mouse diet. The crop, PM and colon were full in all females, presumably indicating that food was still passing backwards into the colon (although at an unknown rate). Brown fluid was observed to fill the ectoperitrophic region of the anterior midgut and caeca but no Azure B was found there. Also, there appeared to be no absorption of dyes on the PM in the ileum. There was no

evidence therefore, of any movement of dye into the alimentary tract of these cockroaches - although such evidence would have been lost if stained PM had already moved into the colon where it would be subject to considerable deterioration (see 2.4.1).

Another five females that had already had access to mouse diet for five days, were injected with 5 µl of 0.5% Azure B at the commencement of the scotophase and dissected 60 minutes later, and five more females were injected at the middle of the photophase with 10 µl 0.5% Trypan blue and dissected 30 minutes later. Only in those cockroaches with an empty crop could dye (Azure B or Trypan blue) be discovered adsorbed onto the PM at the pyloric valve; dye was not found in females with a full crop.

The above results suggest that the Malpighian tubules take up injected dyes and transport them to the ectoperitrophic space in the ileum where dyes are adsorbed onto the ectoperitrophic surface layer, irrespective of whether or not the cockroach had been feeding (Figure 2.3), although in females that had continuous acess to food, injected dyes could not be traced anywhere in the gut, perhaps because rapid throughput of ingesta meant that any excreted dyes or stained PM fragments became intimately mixed with the contents of the colon. In those insects in which dye was observed on the PM in the ileum, it appeared as a well defined band, and it may be assumed that it was adsorbed on to the PM directly under the

ampullae of the Malpighian tubules (Figure 2.3a). This was the only evidence of entry of dyes into the alimentary canal of cockroaches that already had a full gut, but in adult male cockroaches that had been feeding for only 1.5 or 2.5 hours after fasting for two days, injected dyes also appeared in the caeca, sometimes in the ectoperitrophic region of the anterior midgut, and even in the crop (Figure 2.3b). Unless the caeca take up the dye directly from the haemolymph, the presence of injected dyes in the anterior region of the midgut would seem to support the concept of an anteriad flow of fluid, derived from the Malpighian tubules, through the ectoperitrophic space (Figure 2.3b). This evidence is further discussed below.

3.4 **Discussion.**

3.4.1 **Production of the PM.**

Results of adsorption of injected dyes onto the PM in the ileum give an indication of the rate of production of the PM. If dyes were assumed to be adsorbed onto the PM in the ileum directly under the ampullae, then finding them at the pyloric valve as soon as 20 and 30 minutes after injection in some males and females respectively, indicates a rapid production of the PM (6-8 mm/hr). The injection itself must have caused a brief production of PM in the fasting cockroaches, perhaps due to a change in haemolymph osmolarity or volume, since the band of dye was found at the pyloric valve not only 20 minutes after

the injection, but also one and three hours after the injection.

3.4.2 Passage of dyes incorporated in an artificial diet through the gut of P. americana.

Both insoluble components (i.e. silica) and solutes (i.e. dyes) from diets offered to fasted cockroaches were seen in the crop, midgut, and anterior colon, one, two to three, and four hours, respectively, after the diet was first offered (section 3.3.1) These rates of passage for coloured food were in agreement with those reported by Snipes and Tauber (1937) and Snipes (1938) for P. americana feeding on either coloured banana paste or mouse diet.

Cockroaches appear to ingest some of each diet offered (section 3.3.1) with an initial peak of feeding when first offered food and a subsequent peak in some cockroaches on day 3. This agrees with other observations (see Bignell, 1982; Rollo and Gunderman,1984) where adult male P. americana would initially ingest a large meal and then reduce their intake and become more variable in ingestion times and meal size. Rollo and Gunderman (1984) showed quantitatively that the amount male cockroaches ingest on a day to day basis is very variable although there was an apparent peak of feeding in some male cockroaches three days after being transferred from crowded conditions to isolated containers. Also, days with high

consumption tended to be followed by days of low consumption. Snipes and Tauber (1937), Snipes (1938) tried to eliminate some of this variation by depriving the cockroaches of food before presenting it to them again, but found repeated trials with the same test insect were very variable in relation to individual egestion times. Also, cockroaches given food ad lib after being deprived of it for a short period tend to consume larger than normal amounts (in Bignell, 1982).

When different diets were offered to the cockroaches serially, there was obvious mixing of dietary materials in the crop, hence meals were no longer discrete in other parts of the gut once a second diet was ingested; a circumstance that is not uncommon (Cornwell, 1968).

Dyes that were incorporated into diets were found distributed the length of the midgut only when there was a peak in feeding activity. This was also reflected by the distribution of the natural brown pigment in the first four hours after the insects were offered diet 1 (section 3.3.1) and the apparent subsequent staining of the posterior region of the midgut epithelium with dye (Figure 2.1.a - d). At other times, dyes were found only in the anterior region of the midgut and the caeca and tended to remain in this area for long periods; for example both Orange G and 5-aminoacridine (from the second and third diets respectively) were still present in the caeca after clearing of the crop and ingestion of diet 4 (section

3.3.1).

Associated with peaks in feeding is the seemingly increased fluidity of the PM down the length of the midgut; which was assumed to be due to an increase in the rate of synthesis of PM (see Chapter 4).

3.4.3 Location of dyes when injected under various conditions.

Dyes were found adsorbed on the PM in the ileum which gave some estimate of the rate of production of the PM (as discussed in 3.4.1). There appears to be some movement of dyes into the caeca but it is unclear whether it was due to direct absorption from the haemolymph or a countercurrent flow as suggested by Dow (1986), whereby fluid derived from the Malpighian tubules is considered to pass into the ectoperitrophic space and then moves anteriad, counter to the movement of the solid ingesta in the endoperitrophic space. Although the source of the dyes could be the Malpighian tubules, it is difficult to reconcile the distinct banding of the PM in the ileum with the possible anteriad flow of dye; the banding would appear only possible if dyes were confined to a distinct region of the ileum, i.e. directly under the ampullae. Perhaps an initial banding and movement of the PM towards the colon, is separated temporally from any subsequent anteriad

flow of the dye in such a way that the dye at one time remained in contact with the PM immediately under the ampullae long enough to stain the PM but when an anteriad flow occurred, this was too rapid to allow appreciable staining along the full length of the PM. More investigation with strict control on feeding would be necessary for clarification.

3.4.4 Effect of tannic acid incorporated into artificial diet on movement and assimilation of the diet.

The incorporation of 0.5% tannic acid in the diet with various dyes had little immediate effect on the movement of dye or food through the gut (section 3.3.2). By the third day of presentation, however, cockroaches appeared to cease, or slow feeding, holding the food in the crop for a longer period compared with controls.

This might explain the reduced faecal weight observed with increasing tannic acid concentration when cockroaches were fed for five days on diets containing from 0 to 0.5% tannic acid (section 3.3.3). The reduced faecal weight may explain increasing AD seen for increasing tannic acid concentrations. Also whether tannic acid is beneficial at lower concentrations, as suggested by a high ECD for 0.05% tannic acid (Table 3.2) but detrimental at higher concentrations (0.1 and 0.5% tannic acid), as suggested by lower consumption and ECD is unclear at this stage since no comparisons have been made with a complete

diet (eg, mouse food), or for a long enough period or over a number of stadia.

CHAPTER 4 : Production of the PM.

4.1 Introduction.

Lee (1968) described secretion of the PM in P. americana by a ring of well defined anterior midgut cells lying between the start of the oesophageal invagination intima and the openings of the enteric caeca. According to Wigglesworth (1972), this would be a Type II production of PM (by secretion). Lee also claimed there was addition of supplementary laminae by the general midgut epithelium posterior to the caecal bases and proximal sections of the ceacal epithelium. Despite these observations by Lee, there is still reference to so-called Type I production by delamination in cockroaches and orthopteroids in general (e.g. Chapman, 1980), which is the secretion ("delamination") of PM layers from the length of the midgut epithelium (see discussion by Peters, 1976 c.f. Richards and Richards, 1977).

Lee's observations were based on histological studies of the anterior midgut where widely separated laminae from apparently different origins were found in the lumen of the anterior midgut. There appeared to be some differentiation of midgut epithelial cells in this region with cells more elongate and with the brush border being deeper. Further, epithelial cells (whether only of the anterior midgut or not is unclear)

were strongly PAS-positive; minute granules that occurred between the brush border and nuclei were PAS-positive and enzyme-fast. These were considered to be mucopolysaccharide in nature, but whether acid or neutral could not be clearly discerned from histochemical tests. Vacuoles in cells of the anterior midgut (including the caeca and a ring of cells below the caeca) stained strongly for protein. The preceding is suggestive of secretory cells but to conclude they secrete the PM on this evidence alone is perhaps unwarranted. Wigglesworth (1972) warned against presuming a Type II production of PM merely from a close association of PM with the oesophogeal invagination and adjacent midgut tissue; he suggested an alternative "raison d'être of this invagination : it enables the PM to extend forward beyond the point at which the food enters the midgut".

From general observations however, the PM does appear to be produced by cells of the anterior midgut (see Chapter 2) and the way in which it is produced could affect a variety of factors involved in digestion and also the presumed function of the PM in protection of the midgut epithelium (see Chapter 7). It seemed therefore that production of the PM should be reinvestigated in some way other than histological techniques.

Male roaches deprived of food for six days, were injected as described in 4.2.1 with [C14]-N-acetyl-D-glucosamine ([C14]-NAGA), a chitin precursor, and [H3]-cystine dihydrochloride,

which was used as a general label for sulphur-containing protein (referred to hereafter as [H3]-cystine). PMs of Calliphora erythrocephalla are known to contain small amounts of S-containing amino acids (Zimmerman et al., 1973). It was expected that there would be clear differences in the pattern of incorporation of labels if the PM was produced by cells of the anterior midgut or "delaminated" by cells of the general midgut epithelium. Radioactivity was determined by liquid scintillation with further, qualitative evidence from whole PM autoradiography.

Results indicated both labels were preferentially incorporated in anterior PM sections and that the PM is produced by the cells of the anterior midgut. PM production was estimated to be 5-7 mm/hr for at least the first four hours after beginning to feed.

4.2 **Materials and Methods.**

4.2.1 **Insect treatments for liquid scintillation.**

For determination of the rate of production and whether new PM is secreted only by cells of the anterior midgut or by the midgut epithelium generally, adult male cockroaches were readied for feeding as in section 2.1 except that on the fifth day of fasting and four hours before the commencement of the next photophase they were placed at four ^{o}C; two hours later

they were injected through the intersegmental membrane of the seventh abdominal sclerite with 5.29 MBq of [H3]-cystine dihydrochloride (specific activity of 66.6 MBq/mg, Amersham), and 1.81 KBq of [C14]-N-acetyl-D-glucosamine ([C14]-NAGA) (specific activity 9.73 MBq/mg, Amersham); each label in one microlitre of saline. Controls were injected with saline only. Two hours before commencement of scotophase, the roaches were again placed at 27 ^{o} C. A standard diet (section 2.5) was placed in their container at the commencement of the scotophase; water was provided ad lib. Groups of twelve adult males (with two controls) were dissected at various times after the commencement of the scotophase : 0.5, 1, 1.5, 2.0, 3.0, 4.0 hours after first being observed to feed; a control group without access to food was dissected at commencement of scotophase. Any roaches not feeding within an hour of commencement of scotophase were discarded.

4.2.2 **Treatment of gut tissues and PM sections for liquid scintillation counting.**

Haemolymph samples were collected with a two microlitre microcap from a cut made between the intersegmental membrane of the metathoratic femur and coxa; the collected fluid was expelled immediately into PCS fluor (Amersham). Guts (from crop to rectum) were dissected out and, as for all subsequent sections to be assayed, rinsed eight times in saline. Also, about five grams of visceral fat was removed and rinsed, then

placed on a square of aluminium foil. Subsequent manipulations were in chilled wax dissecting dishes, which were changed between insects. Dissected guts were pinned out on the wax, blotted dry and stripped of fat and tracheae. Caeca were torn open with fine forceps emptying their contents onto the wax. Two microlitres of this fluid was collected from each insect with a microcap and expelled immediately into fluor as above.

Chilled saline was added to cover the gut. Caeca were then separated from the main trunk of the midgut, with the latter arbitarily divided into three equal sections, anterior, middle and posterior midgut (antMG, midMG and postMG respectively), and separated from their corresponding PM sections (antPM, midPM, and postPM). The ileum and anterior colon and their corresponding PM sections (ileumPM and colonPM respectively) were also removed. All tissue and PM sections were rinsed in saline, blotted and placed on squares of aluminium foil (as above). Samples were dried to constant weight at 60 ^{o}C and kept desiccated. Weights were determined on a Beckman microbalance. Gut sections were digested with NCS Tissue Solubiliser (Amersham) at 60 ^{o}C overnight; the solutions were cooled, fluor added and mixed vigorously. In order to remove most of the protein from the PM, leaving mainly the chitinous fibrils, sections were treated with 50% KOH (Analytical Reagent grade) for 30 mins at 100 ^{o}C in sealed polypropylene tubes. The residual PM sections after washing with dilute alkali (0.1M KOH) were removed to new polypropylene

tubes. Acetic acid (Laboratory Reagent grade) was added to neutralise any remaining KOH and to dissolve the residue of the PM. Radioactivity of both labels was measured on a Beckman 3801 liquid scintillation counter with dual window mode (75% counting efficiency).

4.2.3 **Autoradiography.**

Insects were prepared for autoradiography as above (section 4.2.1) but injected with [C14]-NAGA only and at half the dose (0.93 KBq) Groups of eight adult male roaches per time interval were used and the label incorporation determined at the commencement of scotophase, and at 0.5 and 3 hours after the roaches had first been observed to feed. Only the PM was needed for autoradiography. It was dissected whole from the gut under the same conditions as above (section 4.2.2). The PM was rinsed eight times in chilled saline. Half the samples per treatment were divided as in section 4.2.2 into antPM, midPM, postPM, ileumPM and colonPM. The individual sections thus divided were added to individual polypropylene tubes and then treated with 50% KOH as above (section 4.2.2) after which they were washed with 90% ethanol and placed on a glass slide. Whole PM lengths untreated with KOH were also placed on glass slides. Both whole PMs and PM sections were dried at 60 ^{o}C in a desiccator, wrapped with thin polyethylene film and overlaid with Fuji "RX" medical grade X-ray film. Another glass slide was taped on top to sandwhich X-ray film against PM sections.

Samples thus packaged were left between lead sheets (connected by copper wire to prevent build up and spark discharge of static electricity) in a lead lined box. The films were developed after a week.

4.3 **Results.**

4.3.1 **Level of labels in body fluids and fat body.**

Table 4.1 shows both labels generally elevated in caecal fluid at the beginning of the study period, and falling to lower steadier levels by two hours after first feeding. Haemolymph had relatively steady labels for both compounds over the four hour period of observation. Similarly, incorporation in fat body was relatively steady over the four hour period. Caecal fluid and haemolymph were noticeably easier to collect one hour after feeding had begun, when there appeared to be a marked increase in volume. Hence the drop in label concentraton in the caecal fluid could be due to dilution from other sources, e.g. fluid from ingesta (see section 4.4)

4.3.2 **Incorporation of labels into gut tissues.**

All results were analysed with Genstat V (Copyright, Rothamstead Experimental Station) analysis of variance. All data were logged to normalise the data set. There was no difference found between the midgut sections midMG and postMG

TABLE 4.1 : Incorporation of labels in body fluids and fat body.

		TIME (hours after first feeding)						
LABEL	FLUID/ TISSUE	0	0.5	1.0	1.5	2.0	3.0	4.0
[H3]		mean log [(ng [H3]-cystine + 1)/g tissue)]						
	Caecal Fluid	2.81	2.60	2.89	2.99	2.30	2.16	2.00
	Haemo-lymph	2.24	2.00	2.11	2.13	2.02	2.16	2.00
	Fat Body	2.77	2.95	2.93	2.90	2.65	2.87	2.71
[C14]		mean log [(ng [C14]-NAGA + 1)/g tissue)]						
	Caecal Fluid	1.60	1.62	1.46	1.41	0.93	0.66	1.12
	Haemo-lymph	0.95	1.04	0.94	0.92	0.78	0.84	0.85
	Fat Body	3.26	3.26	3.16	3.40	3.24	3.24	3.24

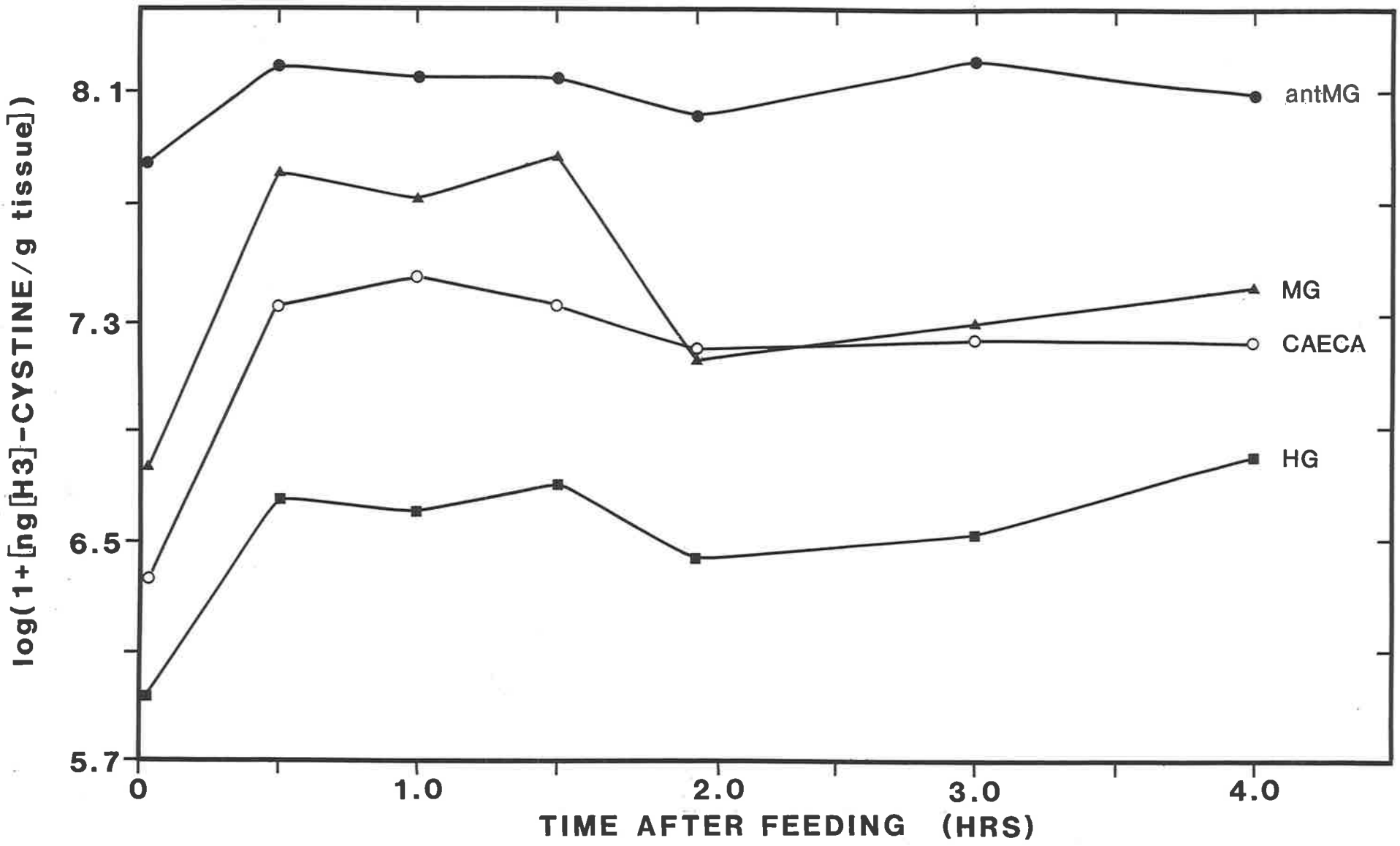
or between the hindgut sections, ileum and colon. It was therefore convenient to pool the data from these sections: they will be referred to as MG and HG, respectively.

Incorporation of the tritiated cystine label into gut sections followed the same trend for each section. A rise in label occurred at 0.5 hours after first feeding, after which the label remained relatively constant over the four hour period of observation. The antMG had the highest incorporation of around 100 mg label/g tissue followed by the rest of the midgut sections. The pooled (middle and posterior) MG sections were only significantly different from the caeca ($P < 0.05$) at four hours after feeding. Hindgut sections had a significantly lower incorporation of [H3]-cystine than the other sections ($P < 0.05$) over the four hours after feeding (Figure 4.1). The preceding suggests that the antMG may utilise the amino acid in a similar fashion to the other gut sections, but that the antMG has the greatest requirement for the [H3]-cystine of the gut tissues studied.

[C14]-NAGA incorporation in the antMG before feeding was much higher compared with the rest of the gut sections, including the caeca. Thereafter, [C14]-NAGA incorporation in the antMG was similar to the caeca. The caeca showed constant incorporation for the course of the study (approx. 1.6 ug label/g tissue). The pooled MG sections followed a similar trend to that of the antMG but at a significantly lower level ($P < 0.05$) during the period under observation. There was a

Figure 4.1

[H3]-cystine incorporation in midgut tissue.


log(1+[ng[H3]-CYSTINE/g tissue])
8.1
7.3
6.5
5.7
0
1.0
2.0
3.0
4.0
TIME AFTER FEEDING (HRS)
antMG
MG
CAECA
HG

constant increase in the incorporation of label in hindgut sections with levels increasing from a very low level of incorporation of 15 ng [C14]-NAGA /g tissue immediately after feeding to a level of incorporation not significantly different from the MG sections (Figure 4.2).

There was therefore an apparent general utilization of [H3]-cystine by all gut tissues compared with a more specific utilization of [C14]-NAGA by midgut tissues. Further, in the case of both labels it appeared that the antMG had the highest incorporation of label.

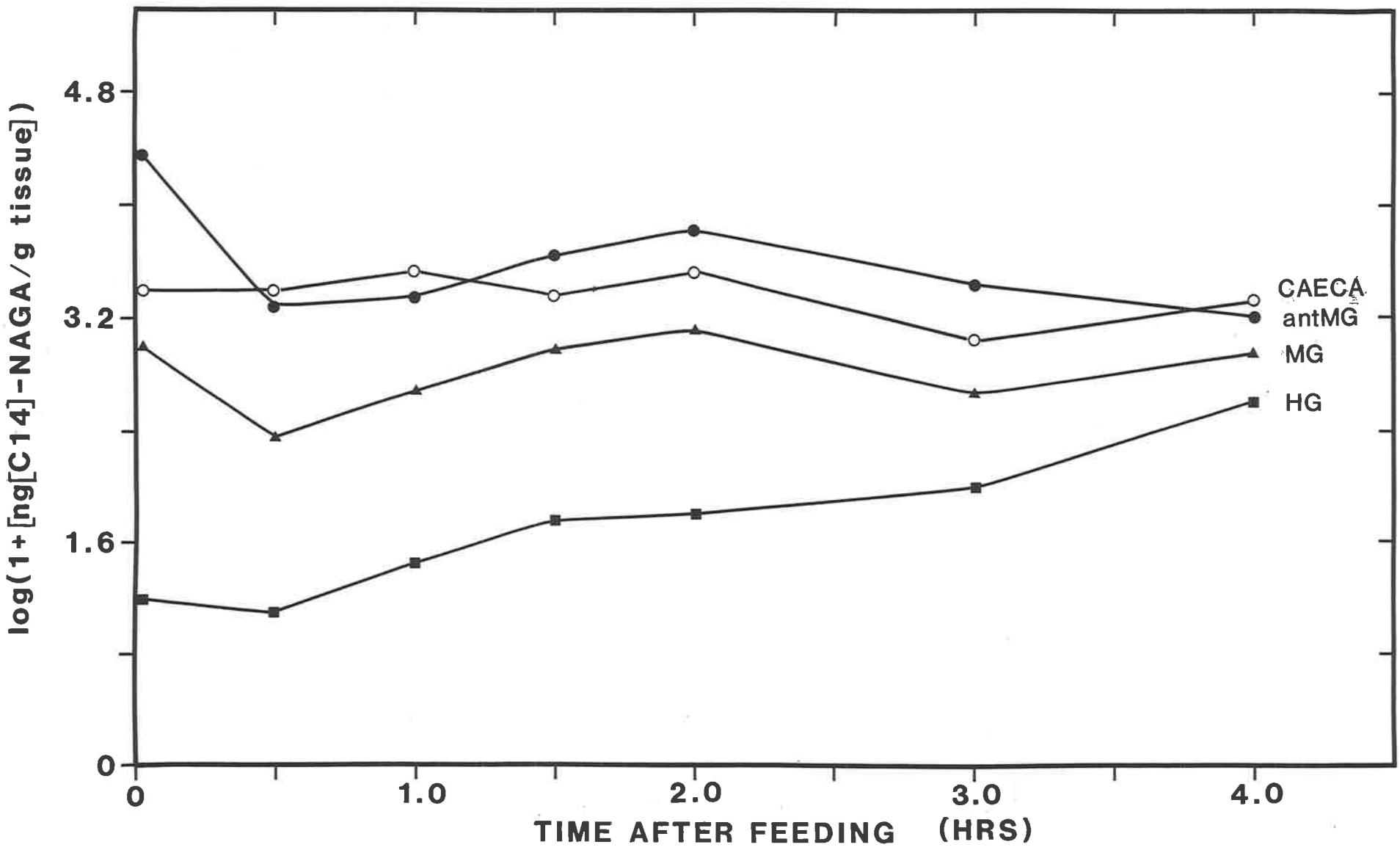
4.3.3 **Incorporation of labels into the PM.**

The analysis of results was as in 4.3.2. Alkali extracts, with few exceptions, followed the same trend over the time period studied as the residues of alkali treated PM sections, so the data were combined. Further, this was considered also to reduce the influence of artifacts caused by removal of any structural components due to the severe treatment with hot alkali. The alkali extracts were considered important, however, since they would have shown any relative differences of solubilities of components, and hence their relative significance in the structure of the PM.

Sections of PM from the colon when observed had no measurable radioactivity and were too few in number to include

Figure 4.2

[C14]-NAGA incorporation in midgut tissue.


log(1+[ng[C14]-NAGA/g tissue])
4.8
3.2
1.6
0
0
1.0
2.0
3.0
4.0
TIME AFTER FEEDING (HRS)
CAECA
antMG
MG
HG

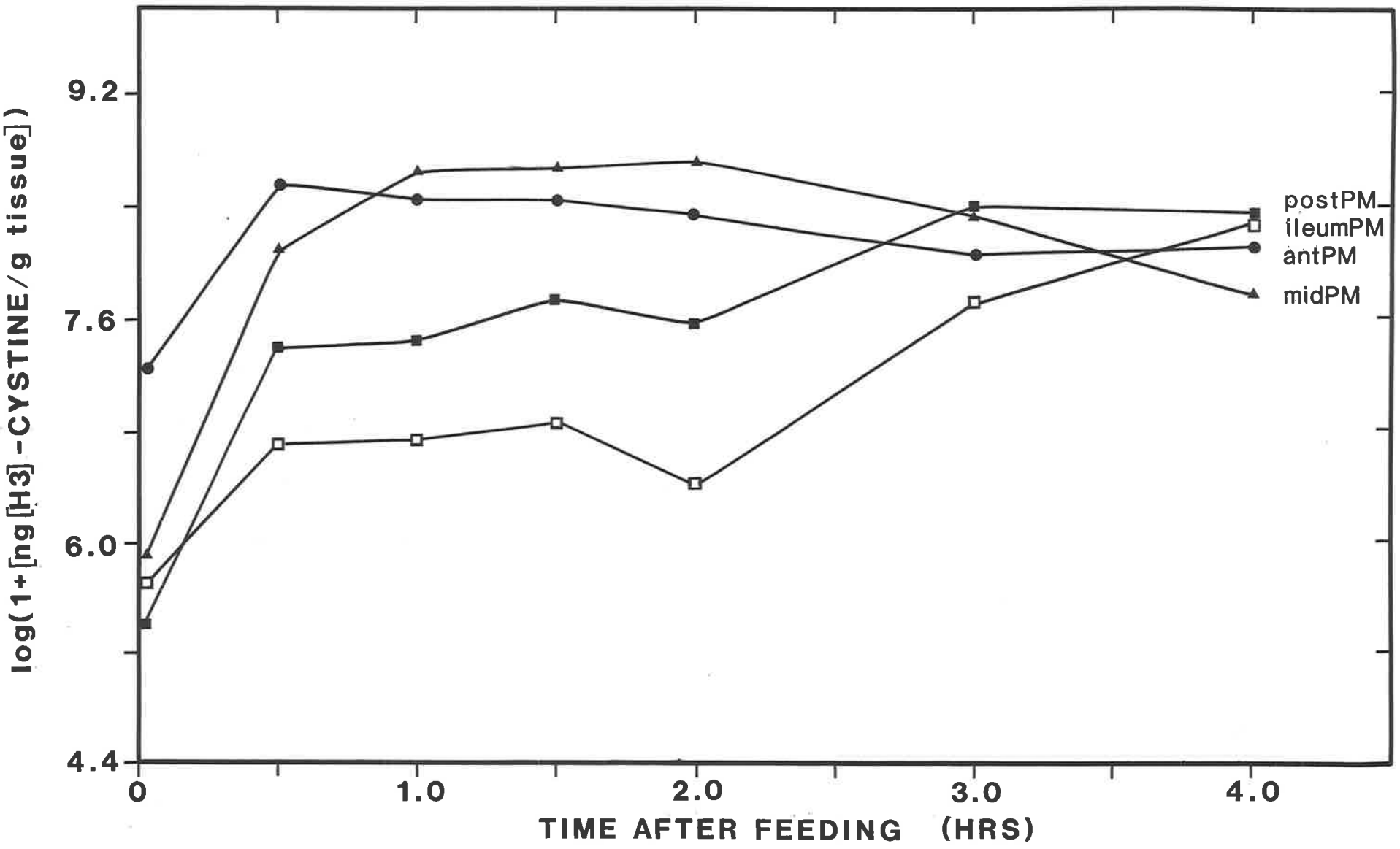
in subsequent analyses.

Figure 4.3 shows that [H3]-cystine reached a peak of incorporation in the PM that apparently moved steadily down the gut. Thus [H3]-cystine incorporation before feeding was significantly higher in the antPM compared with other sections. An initial 16 mg [H3]-cystine/g membrane rose to 360 mg [H3]-cystine/g membrane 0.5 hours after feeding and fell slightly to 120 mg [H3]-cystine/g membrane. The midPM showed a similar trend although starting from a much lower level and reaching maximum incorporation one hour after feeding. Incorporation of [H3]-cystine in the postPM increased from the beginning of the time period, until by three hours after first feeding, it had reached a level comparable with the elevated levels of the antPM and midPM. Similarly the ileumPM reached levels of [H3]-cystine incorporation comparable with the elevated values of the other sections by four hours after first feeding.

Incorporation from cystine into the PM appear to be very high and may have been overestimated as discussed at the end of this chapter. Alternatively, because of the relatively high level of [H3]-labelling in all samples, it is possible that the data indicate both incorporation into the structure of the PM as first secreted, together with a subsequent, more general adsorption of label from tracer diffusing into the gut along the whole its length. Nevertheless, results showed consistent trends and may be considered to give, at the very least, an

Figure 4.3

[H3]-cystine incorporation in PM sections.


9.2
7.6
6.0
4.4
log(1+[ng [H3]-CYSTINE/g tissue])
0
1.0
2.0
3.0
4.0
TIME AFTER FEEDING (HRS)
postPM
ileumPM
antPM
midPM

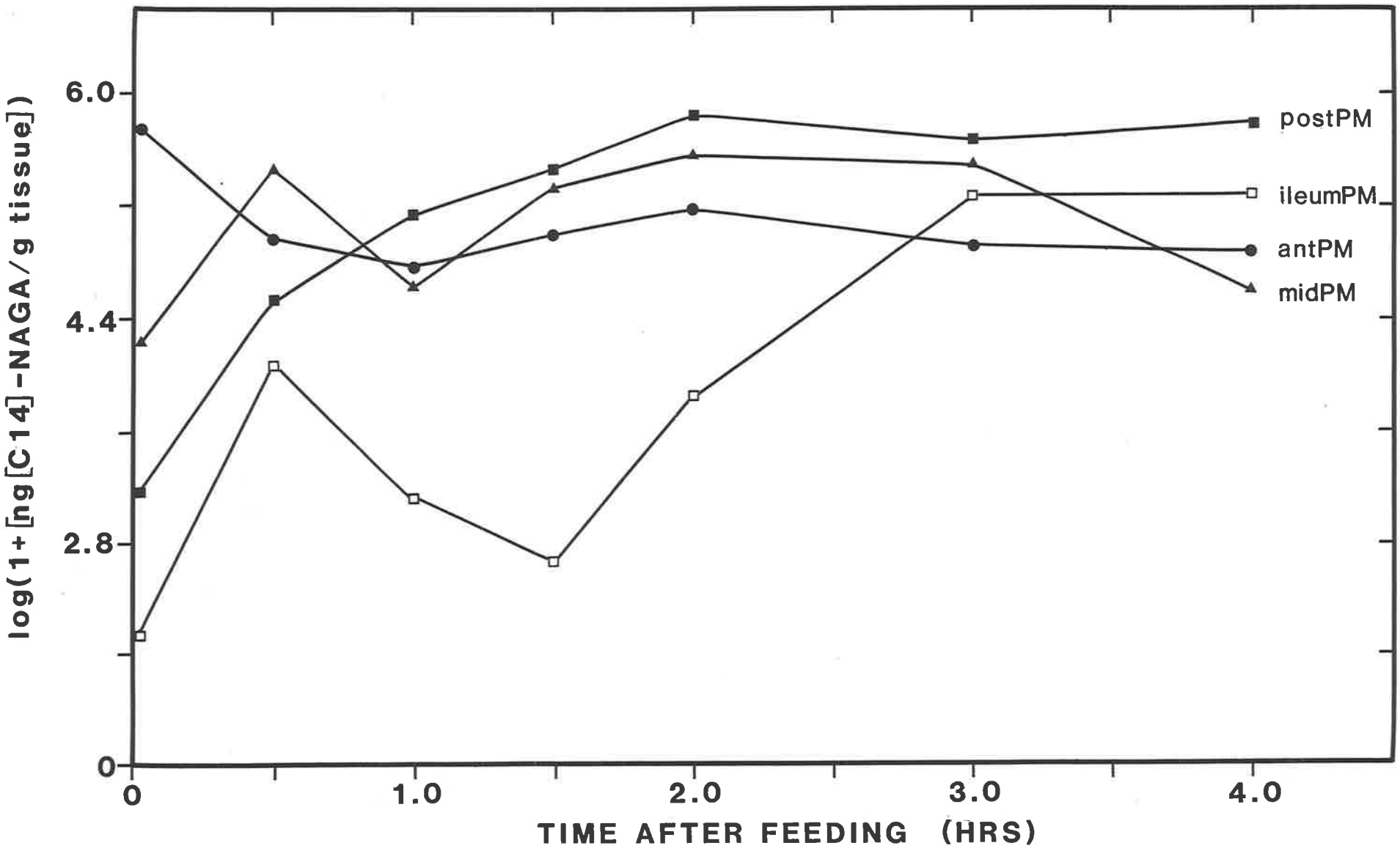
indication of changes of relative activities in different parts of the PM with time.

The significant difference between the antPM and other sections of the PM immediately after feeding and the increase in incorporation of label in the midPM and postPM to a level similar to that of the antPM suggests the label is utilised in PM production in the anterior and that the membrane then moved along the length of the midgut and into the ileum; i.e. by four hours after first feeding, a newly secreted membrane had occupied the entire length of the midgut and ileum. Given that the total length of this region of the gut was 18 mm, then the rate of production of PM would have been approximately 5.5 mm/hr.

From Figure 4.4 it is apparent that peak incorporation of [C14]-NAGA followed the same trend as that of labelled cystine although levels of incorporation tended to fall off once the peak had passed. Incorporation before feeding was highest in the antPM i.e. 0.55 mg [C14]-NAGA/g membrane which was significantly different from other sections ($P < 0.05$). It then fell to around 0.10 mg [C14]-NAGA/g membrane for the rest of the time period (Figure 4.4). The midPM rose markedly at 0.5 hours after feeding from 17 ug to 280 ug [C14]-NAGA/g membrane. It remained at this level until 4 hours after first feeding when it fell to 36 μg [C14]-NAGA/g membrane, although there is an apparent decrease one hour after feeding which seems

Figure 4.4

[C14]-NAGA incorporation in PM sections.


log(1+[ng[C14]-NAGA/g tissue])
6.0
4.4
2.8
0
0
1.0
2.0
3.0
4.0
TIME AFTER FEEDING (HRS)
postPM
ileumPM
antPM
midPM

anomalous and could simply be a deviant estimate (Figure 4.4).

[C14]-NAGA incorporation in the postPM rose from a low level of 1.4 ug to 7.1 μg [C14]-NAGA/g membrane during the first 2 hours after first feeding ($P<0.05$), and did not deviate significantly from this level during the last 2 hours after feeding.

Incorporation of [C14]-NAGA in ileumPM sections also rose from a low level but with a marked peak at 0.5 hours after first feeding, then fell in the next hour. The initial rise is difficult to explain unless assumed to be due to contamination through excretion of label via the Malpighian tubules (see 4.4). Incorporation increased thereafter reaching an elevated level of incorporation comparable to maxima of other PM sections.

As with incorporation of [H3]-cystine, the significantly higher level of incorporation of [C14]-NAGA in the antPM immediately after first feeding, followed by a marked increase in the incorporation of [C14]-NAGA in sections posterior to the antPM, sequentially down the length of the PM following the beginning of feeding, suggests that [C14]-NAGA is incorporated preferentially at the anterior end of the midgut, i.e. that it is secreted there. It appears that a newly secreted membrane occupies the length of the midgut and ileum by three hours after the beginning of feeding implying a rate of production

(given that the total midgut and ileum length from which sections were taken was approximately 18mm) of approximately 6mm/hr, which agrees closely with the estimate obtained from [H3]-cystine incorporation.

4.3.4 **Alkali treatment of PM sections.**

Alkali treatment of the PM would have removed much of the protein fractions and presumably any materials adsorbed onto or incorporated into the PM after its secretion leaving mainly insoluble chitosan. A low proportion of label in the alkaline extract could therefore be interpreted as indicating that most of the label had been incorporated into the chitin or was strongly bound to the structural chitin of the PM. Total levels are indicated in Figures 4.3 and 4.4 while Table 4.2 shows the percentage of label in the alkali fraction.

As might be expected, there was considerable solubilisation of label derived from [H3]-cystine from all PM sections, which was almost total from the ileumPM for the first 1.5 hours after feeding. Also, the proportion of label extractable from the antPM increased once the roach had begun to feed (Table 4.2).

There was a high degree of variability for alkali extractions of label originating from [C14]-NAGA. Nevertheless, there appeared less solubilisation of [C14] compared with the

TABLE 4.2 : %Label in alkali fraction (OH) of PM sections.

LABEL	PM SECTION	TIME (hours after first feeding) 0	0.5	1.0	1.5	2.0	3.0	4.0
[H3]		[OH /(PM + OH)] x 100*						
	APM	38.0^{a}	81.8^{a}	76.1^{a}	77.7^{a}	72.1^{a}	85.5^{a}	77.2^{a}
	MPM	37.2^{a}	64.8^{b}	48.1^{bc}	60.6^{a}	64.3^{a}	76.5^{ab}	80.2^{a}
	PPM	53.4^{a}	59.0^{b}	59.5^{c}	60.2^{a}	63.5^{a}	65.8^{bc}	73.0^{a}
	IPM	97.8^{b}	98.4^{c}	82.4^{d}	79.9^{a}	63.8^{a}	67.4^{bc}	84.2^{a}
[C14]		[OH /(PM + OH)] x 100*						
	APM	15.7^{a}	41.0^{a}	37.1^{a}	30.8^{a}	36.0^{a}	39.3^{ac}	67.1^{a}
	MPM	19.0^{a}	23.3^{b}	30.8^{a}	25.5^{a}	33.7^{ab}	44.8^{a}	44.9^{b}
	PPM	23.5^{a}	34.5^{a}	12.4^{b}	13.8^{a}	20.8^{bc}	16.7^{bc}	36.5^{b}
	IPM	10.0^{a}	56.1^{a}	41.6^{a}	60.1^{b}	37.4^{a}	24.5^{c}	71.8^{a}

a,b,c : different superscript within columns imply significantly different ($P<0.05$, Results analysed with ANOVA, Genstat V, Rothamstead Experimental Station).

* : specific activity of alkali fraction as a percent of total specific activity of PM section + alkali fraction.

[H3] label : [H3]-cystine dihydrochloride (Amersham).

[C14] label : [C14]-N-acetyl-D-glucosamine.

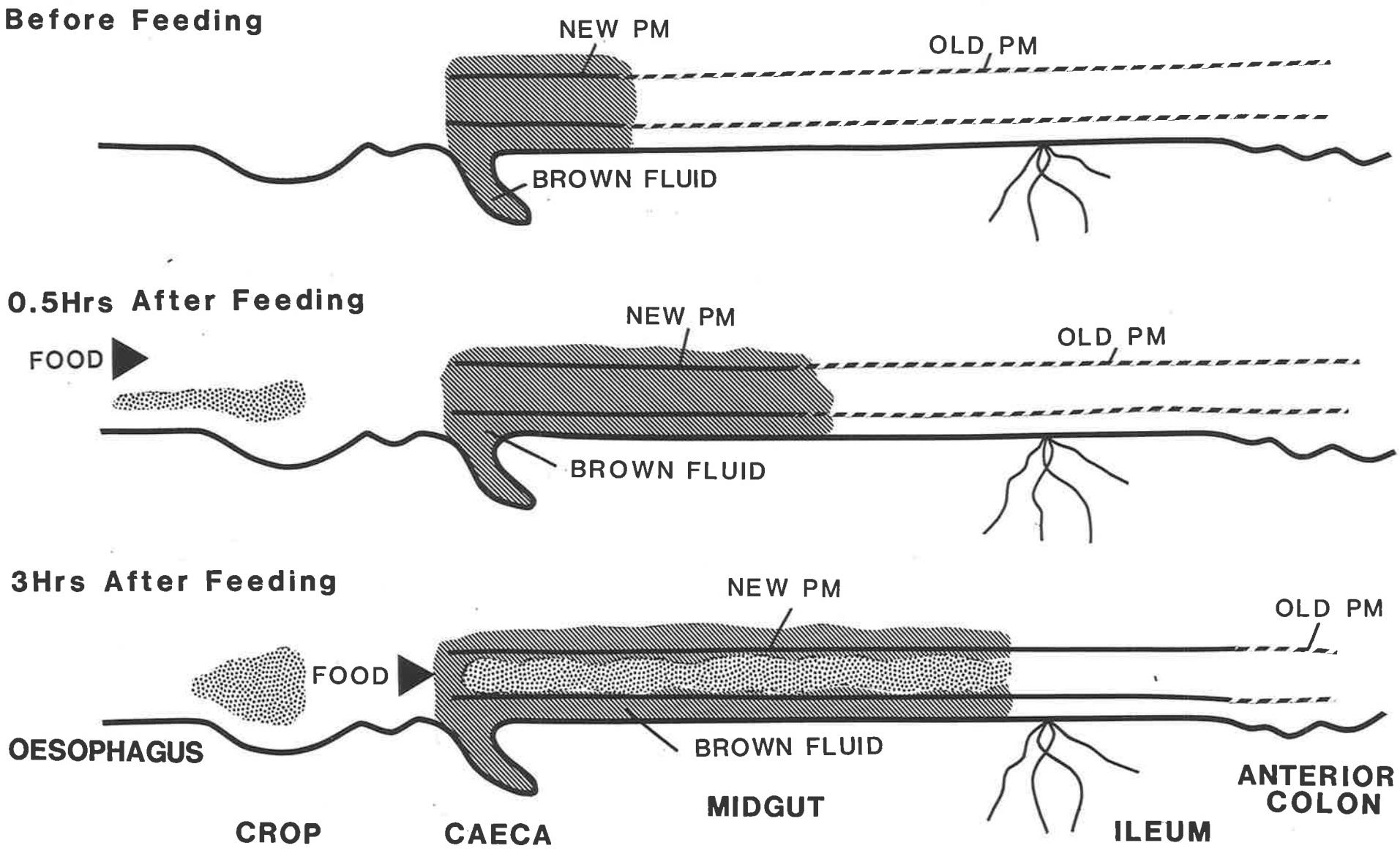
[H3] from cystine. At the same time, whereas the extractability of the label originating in cystine was similar throughout the experiment, the proportion of [C14] label from NAGA that was extractable rose noticably after feeding had commenced, particularly in the antPM and ileumPM. These results are difficult to interpret in detail since alkali would presumably have solubilised a variety of compounds other than chitin, but they do provide evidence that label was adsorbed onto or incorporated into the PM after its initial secretion, mainly in the vicinity of the caeca and posterior to the Malpighian tubules.

4.3.5 **Autoradiography of whole PM lengths.**

Figure 4.5 summarises the sequence of events for roaches injected with only [C14]-NAGA. PM sections that were subjected to alkali treatment showed similar results to whole PM lengths, although with much greater contrast between areas of marginal and substantial incorporation. The label appeared to be incorporated into the antPM sometime between injection of the label and before first feeding. It was continuously produced and the labelled PM moved posteriorly until by 3 hours after first feeding, a newly secreted PM filled the length of the midgut and ileum, with some even in the colon. At this time also, there were definite traces of solid ingesta in the PM lumen (Figure 4.5). The rate of production taken directly from PM lengths and film exposure gave an estimate of 7mm/hr.

Figure 4.5

Movement of food through the gut and diagramtic representation of autoradiography results. Broken line represents PM in the gut before feeding had commenced and which had no [C14]-label. Solid line represents newly synthesised PM with [C14]-lablel. Hatching represents brown fluid.


Before Feeding
NEW PM
OLD PM
BROWN FLUID
0.5Hrs After Feeding
FOOD
NEW PM
OLD PM
BROWN FLUID
3Hrs After Feeding
NEW PM
OLD PM
FOOD
BROWN FLUID
OESOPHAGUS
MIDGUT
ANTERIOR COLON
CROP
CAECA
ILEUM

4.4 **Discussion.**

Results from both liquid scintillation of PM sections and autoradiography of whole PM lengths indicated that after injection, both [C14]-NAGA and [H3]-cystine were incorporated as a pulse that moved posteriorly through the midgut to the colon for at least four hours after first feeding. Had the PM been delaminated, then similar levels could have been expected along the entire length of the PM within a very short time. On the contrary, however, significant differences in incorporation were apparent between sections and these varied sequentially with time. Further, a rate of production of the PM can be estimated from the data of between 5-7 mm/hr under the conditions of the experiments, one that lies within the range reported for other insects (see Richards and Richards, 1977). Although the evidence may not provide definitive support for secretion of the PM by cells of the anterior midgut, there was clearly a higher initial level of incorporation of [C14]-NAGA and [H3]-cystine in this part of the midgut. Possibly a final resolution of the question would have been obtained with TEM-autoradiography.

[C14]-NAGA is incorporated in the antPM (55 ug [C14]-NAGA/g tissue) at a significantly greater rate than in all other sections ($P<0.05$) before feeding. This initial labelling of the antPM also had a low solubility (16%) in alkali and could therefore be considered as due to

incorporation into structural elements of the PM. This substantial degree of incorporation of [C14] label (and even [H3] label) in the antPM before feeding had even started could be due to stimulation of PM production by the injection of the labels, perhaps due to sudden changes in haemolymph volume and/or osmolarity.

The apparent peak of incorporation of [C14] label half an hour after feeding in at least some IPM sections (there was an associated large standard deviation) is perhaps explicable by observations that dyes injected into starved roaches were apparently taken up by the Malpighian tubules and then excreted into the lumen of the ileum and adsorbed onto the PM (Chapter 3). That this is the case is supported by the higher solubilities for both labels in strong alkali at and around this time. There was also an increase in the incorporation of [C14] label in the hindgut. This did not necessarily reflect incorporation into the cuticular intima of the hindgut, however, since by 4 hours after feeding, [C14]-NAGA could have been metabolised to [C14]-glucose and become generally available to all tissues.

Although waves of incorporation of label from NAGA and from cystine are discernible in Figures 4.3 and 4.4, the peaks appear to be imposed on relatively high levels of background labelling, which are apparent even in the data for the initial samples taken before roaches had access to food. There are

several possible explanations for these high background levels. For [H3] from cystine in particular, they were probably associated with errors due to the low counts of dpm obtained, i.e. just within limits of counting reliability, the low weights of the PM sections, and the well known lability of [H3]. Also, a less than perfect resolution of maximum activities for both labels as they moved down the gut may be due, in part, to the arbitrary divsion of the PM into sections. Fortunately, such problems did not attend the autoradiography data which considerably clarified and definitively supported the sequential incorporation of [C14]-NAGA into the PM.

Food appeared to enter the midgut after a newly secreted PM had filled its entire length. The filling of the PM after injection appeared somewhat slower compared with earlier observations when cockroaches were fed diets containing dyes (Chapter 3), and could be due to trauma associated with the injection since both controls and treatment were affected.

The observation of a high level of radioactivity in caecal fluid before feeding is of interest since this corresponds with the presence of the concentrated brown fluid in the caeca, a fluid that was also intimately and conspicuously associated with apparently newly secreted PM. Lee (1968) also noticed this relationship, and the fluid could be similar in nature to the brown fluid in locusts which is presumed to be a secretory product rich in enzymes (see Baines, 1979). Dow (1981a)

suggested that it was largely the end products of digestion, but from the large number of dissections that were carried out in the course of this experiment, the brown fluid always appeared to be associated with freshly secreted, relatively fluid PM, suggesting a role in PM formation and/or simultaneous enzyme secretion in P. americana.

Generally, the results appear to support Lee in claiming that the PM is by Type II production.

CHAPTER 5 : Morphology of the PM.

5.1 Introduction.

PMs are variform, multilamellate sheaths surrounding the ingesta in most insects with their structural component being chitinous microfibrils (Peters, 1976, 1982; Richards and Richards, 1977). Collections of microfibrils in PMs fall into three categories of networks, or meshworks (Peters, 1976; Richards and Richards, 1977): 1) a random or feltlike network of microfibrils; 2) hexagonal network with fibrils 60 degrees to each other; and 3) orthogonal fibrils at 90 degrees to each other. According to Mercer and Day (1952), the meshwork of microfibres in the PM of P. americana has fibrils at 60 degrees to each other giving a hexagonal orientation. They also reported minor irregularities and unorganised microfibrils embedded in protein ground substance. Peters and Kalnins (1985) described ectoperitrophic surface layers with immobilised aminopeptidases in P. americana. Using both Scanning and Transmission Electron Microscopy (SEM and TEM respectively) the ectoperitrophic surface was examined for any obvious pores or differences along its length.

The effect of tannic acid and gallic acid was also of interest since in vitro tannin-protein complexes are well known and widespread (see Bernays, 1981a). There has been increasing awareness, however, that in vitro conditions do not necessarily

reflect *in vivo* systems to any great degree (e.g. Bernays, 1981a). One of the reasons that a widespread and general role has been attributed to tannins is that little is known about what conditions *in vivo* prevent them from forming complexes (Bernays, 1981a). One often mentioned prohibitive property in some insect guts (noticably lepidopterans) is a high gut pH of around nine (e.g. Berenbaum, 1979). There is increasing evidence lately, however, that tannins do not deserve the status they once had of general, all-purpose, quantitative antidigestive defensive chemicals (Martin *et al.*, 1985). They may have quite different effects, for instance inducing structural changes that increase the susceptibility of proteins to tryptic hydrolysis (Mole and Waterman, 1985). Also they may be rendered harmless by novel mechanisms in some insects, for example tannin-protein comlexes have been shown to be prevented forming *in vivo* due to action of endogenous surfactants (Martin *et al.*, 1985).

Bernays and Chamberlain (1980) have shown that 70% of tannic acid ingested by *Schistocerca gregaria* can be hydrolysed to gallic acid and glucose, and a further 20% of the ingested tannin could be bound to the PM. It was therefore of interest to feed adult cockroaches different diets, including diets containing tannic or gallic acid, and then examine their PM for any changes in morphology.

Results indicated that both tannic and gallic acid had an

effect *in vivo* when ingested by the cockroach, and *in vitro* when the PM was soaked in a solution of either - tannic acid had the greater affect in both cases. Such effects were superimposed on natural differences along the length of the PM in ectoperitrophic surface morphology. It seemed possible, therefore, that the permeability of the PM might be affected by tannic acid, both *in vitro* and *in vivo*, in relation to the position of the PM in the midgut.

5.2 Materials and Methods for studying PM Morphology.

5.2.1 Insect treatments.

For comparison of the effects of ingestion of different diets, with or without polyphenols, adult male cockroaches were readied for feeding as in section 2.2. Groups of four or more adult males per treatment were fed for three days on one of the following: mouse diet (Milling Industries, Mile End), a standard diet (see section 2.2), standard diet with 0.5% (dry weight) tannic acid or 0.5% (dry weight) gallic acid, and then readied for TEM. Other groups of four adult males were fed a standard diet (as above), or standard diet with casein (20% dry weight, fat and vitamin free, Merck), or standard diet with 0.5%, 0.01%, or 0.05% (dry weight) tannic acid, or standard diet with 0.5% (dry weight) gallic acid, and then readied for SEM. Guts were dissected out for electron microscopy in cold 2.5% glutaraldehyde. All the above diets with the standard diet

as a base had a pH of about 6 as measured with a range of indicator papers (Merck). In addition, one group of four adult male cockroaches was fed 0.5% tannic acid (dry weight) incorporated into the standard diet adjusted to pH 3 with acetic acid.

5.2.2 **TEM of the PM.**

To preserve the integrity of the PM as much as possible, the PM was kept in the midgut which acted as a protective sheath through the subsequent handling. The midgut and contents were post-fixed in 2.0% osmium tetroxide after fixing in glutaraldehyde, then stained with 1% phosphotungstic acid. Glutaraldehyde and osmium tetroxide solutions were made up in 0.1 M cacodylate buffer (ph = 7) and the midgut was rinsed between reagents with cacodylate buffer. After the final cacodylic buffer rinse, the midgut and contents were dehydrated in an alcohol series and further stained with warm uranyl acetate in 70% ethanol (modified from Becker, 1978) The midgut was arbitarily divided into anterior and posterior sections and embedded in Spurrs' resin (Structure and Probing, Queensland). Sections were cut on a LKB 4 ultramicrotome using a glass knife and then veiwed with a JEOL 100CX or 100S TEM.

5.2.3 **SEM of the PM and oesophageal invagination.**

The orientation of the PM was maintained in SEM by

dissecting the PM together with the oesophageal invagination. Care was taken to ensure that the positive hydrostatic pressure of the midgut was first released by removing some caeca because it was found that if the PM was exposed without doing so, it would slip off the oesophageal invagination and move posteriorly along the midgut. While still immersed in glutaraldehyde, the PM and oesophageal invagination were manouvered onto an aluminium SEM stub which was carefully lifted out of the glutaraldehyde (Adang and Spence, 1983). The samples were carefully swabbed to remove excess fluid and then fixed to the stub with polycyanoacrylic glue. Since this glue spreads quite easily along any aqueous interfaces, care had to be taken with respect to placement and quantity applied.

Samples were dried by critical point dehydration in a Polarion Critical Point Drier after initial dehydration in an alcohol series. Amyl acetate was not used after the alcohol series and subsequent washing with liquid CO2 was therefore prolonged. Dried samples were coated with 15 nm of gold/palladium in an evaporative coater to minimise colloidal effects (Beaton and Filshie, 1982). A Phillips 500 SEM was used for micrography of samples.

5.2.4 **Fracturography of the PM.**

To obtain some idea on the fluidity (as opposed to the brittleness - see section 2.3) of the anterior versus the

posterior sections of the PM, male cockroaches were fed the standard diet and dissected out as described in 5.2.1 but in physiological saline (as in section 2.2). The sections were then held firmly at either end with forceps and pulled apart with the tensile stress along the longitudinal axis of the PM. When doing this there was an obvious plastic flow in the anterior section of the PM with the PM streaming in the direction of the applied force while the posterior section had a clean brittle break, tearing cleanly at 90 degrees to the applied force.

5.2.5 **Carbon casts of the PM ectoperitrophic surface layer.**

A thin coat of Au/Pd was used to obtain greater detail of the ectoperitrophic surface layer while minimising any artifacts that might have occurred due to the colloidal properties of gold (see section 5.2.3). To overcome this problem even further, a carbon cast was made of the ectoperitrophic surface layer by coating PMs after critic1 point drying with a thin carbon coat on a revolving and tilting stage in a evaporative coater. The PM was removed from the aluminium stub with a thin razor blade and then digested with concentrated HC1. The carbon film was floated onto a copper grid and viewed with a Phillips 100S TEM. The PMs were taken from animals which had fed on standard diet for three days (as in section 5.2.1) and were prepared for SEM as in section

5.2.3.

5.2.6 Saturation of PMs with tannic or gallic acid *in vitro*.

Adult male cockroaches were prepared for feeding as in section 2.2 and given access to standard diet and water for three days. PMs were removed as described before (section 2.2) and the contents washed from them. They were left for four hours in solutions of tannic and gallic acid of varying concentration and pH: 5.0 mM, 25.0 mM, and 50.0 mM at pH 3, 7, 9 or 11 (except that tannic and gallic acid did not dissolve completely when the high concentrations were attempted in neutral solutions - see below). All solutions were made up in phosphate buffered saline (8.0 g NaCl, 0.2 g KCl, 0.2 g KH_2PO_4, and 1.15 g Na_2HPO_4 in 1 l of water) and pH was adjusted with either 1 N HCl or 1 N NaOH after the addition of the polyphenols.

5.3 Results.

5.3.1 General Description of the PM.

There appears little variation in the number of grids along the length of the PM or due to ingestion of different diets (Table 5.1). There was no affect of ingested protein on the morphology of the PM, and only a slight difference in the

TABLE 5.1 : Variation in number of grid layers when roaches are fed different diets.

DIET :	CONTROL	+0.5% TANNIC ACID	+0.5% GALLIC ACID	+PROTEIN
	mean number of grid layers (SE)			
GRID SECTION				
antPM :	15 (4.4)	11 (1.9)	15 (3.2)	13 (6.8)
postPM :	12 (2.3)	12 (6.9)	15 (3.0)	15 (5.7)

morphology of the ectoperitrophic surface layer when cockroaches ingested polyphenols (see 5.3.2). The subsequent discussion can therefore be considered a general description of the PM, regardless of the diet of the cockroach. In cross-section, the PM can be seen to consist of a series of lamellate grid systems and, in lengths of PM from the mid and posterior section of the midgut, an ectoperitrophic surface layer (Figure 5.1). This surface layer has a distinct granular appearance (except when the cockroach ingested diet containing tannic acid - see below) and is closely associated with one (or perhaps two) other microfibrillar layers nearest the ectoperitrophic space (Figure 5.2). Grid layers nearest the endoperitrophic space appear thicker than the rest of the grids; usually about 6 grids had this appearance and when they were slightly turned in sections they showed a regular hexagonal grid system (Figure 5.3) not unlike the other hexagonal grid systems (see below).

At its anterior end, the PM is closely associated with the oesophageal invagination, perhaps due to numerous small spines, microtrichia, on the surface of the oesophageal invagination (Figures 5.4 and 5.5). These have an average density of around 13 per 1000 μm^2, and are roughly conical in shape with the diameter at their base about 0.75 μm and height 1.7 μm. The surface layer of the PM near the oesophageal invagination consists of a loose network of microfibrils in a relatively large amount of ground substance (Figures 5.6a, 5.6b and 5.7) and posteriorly, the whole gradually grades into a

Figure 1 : Cross-section of a length of PM from near ileum-midgut junction, including the endoperitrophic (En) and ectoperitrophic (Ec) space and ectoperitrophic surface layer (indicated by arrow). Scale = 0.1 μm.

Figure 2 : Higher magnification of surface layer (indicated by arrow) interfacing with ectoperitrophic space (Ec). Scale = 0.1 μm.

Figure 3 : Higher magnification of grid layers near the endoperitrophic space (En). Arrow indicates one grid layer rotated, showing a hexagonal orientation of fibrils. Scale = 0.1 μm.

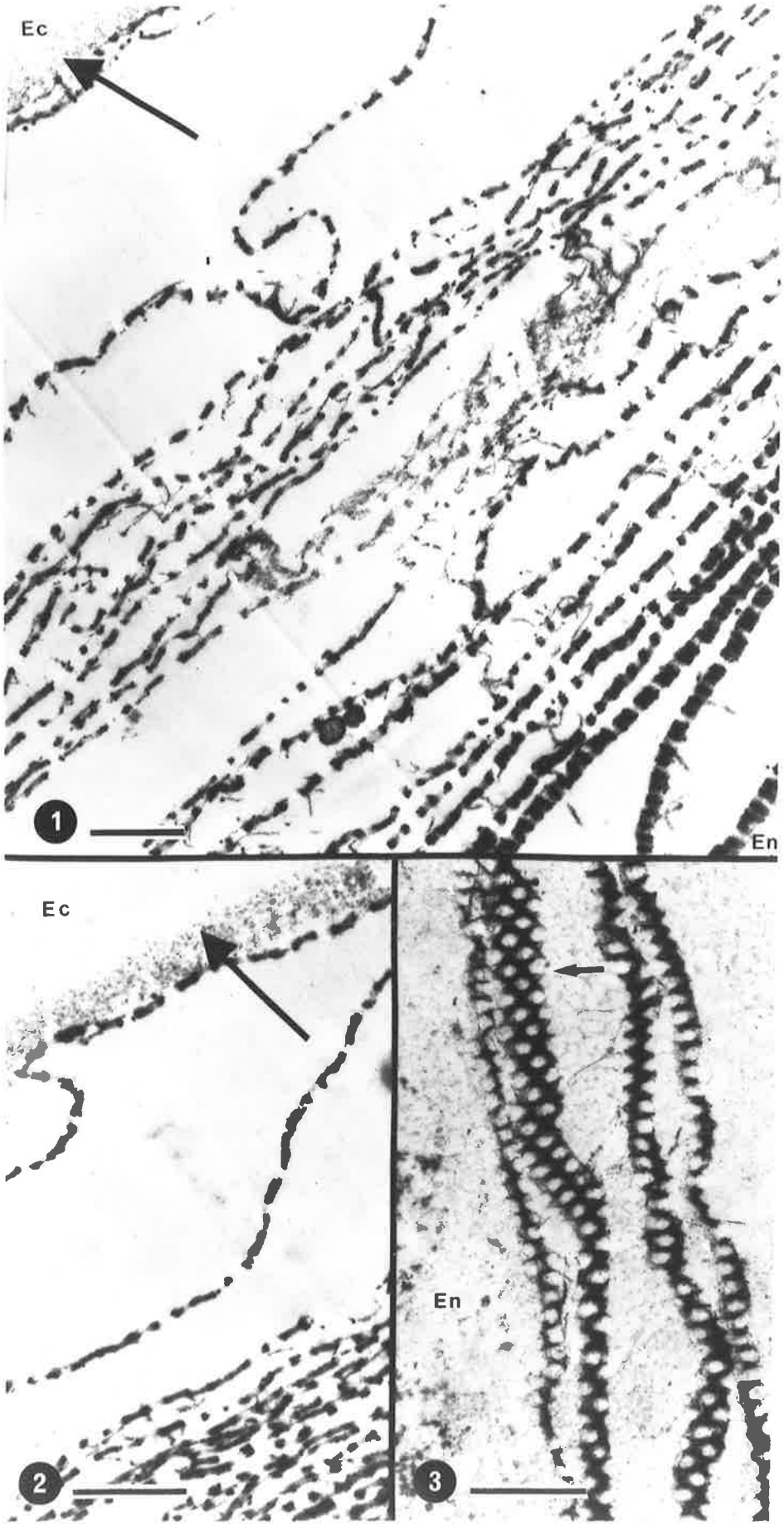

Ec
1
En
Ec
En
2
3

Figure 4 : Surface of the oesophogeal invagination covered with microtrichia. Scale = 10 um.

Figure 5 : Higher magnification of a microtrichium on the surface layer of the oesophogeal invagination. Scale = 1 um.

Figure 6 - 7 : Short microfibrils and ground substance overlying a ectoperitrophic grid layer adjacent the oesophogeal invagination. Figure 6a : Scale 1 um; Figure 6b and 7 : Scale = 0.3 um.

Figure 6a : Disorganised microfibrils with underlying grid sytem.

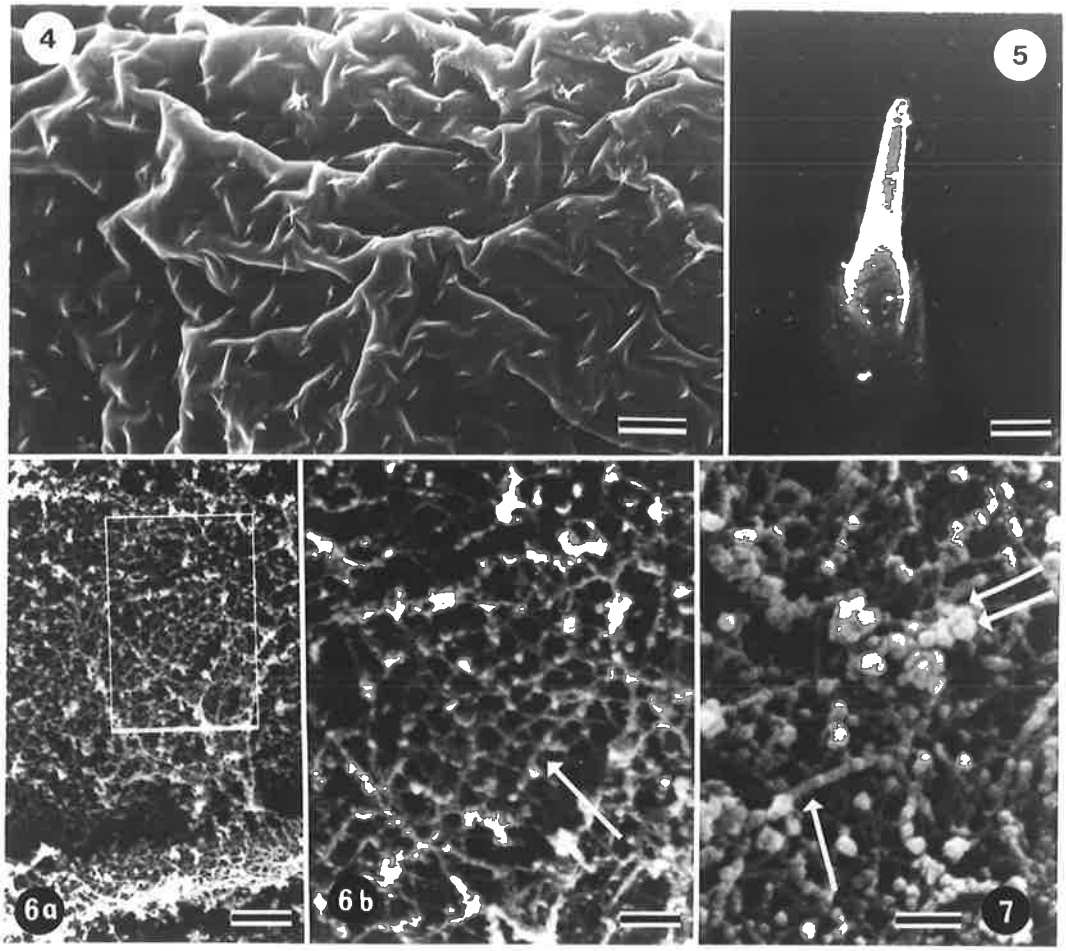
Figure 6b : Inset of 6a; arrow indicates underlying grid system.

Figure 7 : Area slightly anterior to that shown in Figure 6a with a heavier deposit of disorganised microfibrils (single arrow) and ground substance (double arrow).

Figure 8 : Ectoperitrophic grid surface layer showing a largely fibrilar hexagonal orientation. Arrows indicate pores where single fibrils transverse the pore. Scale = 1 um.

Figure 9 : TEM of a carbon cast of an ectoperitrophic grid surface layer and at least one underlying grid system showing their fibrilar nature. Arrow points to junction of fibrils in one layer. Scale = 20 nm.

Figure 10 : TEM of a carbon cast of an ectoperitrophic grid surface layer showing pore, surrounding fibrils and a fibril transversing the pore. Scale = 4 nm.


4
5
6a
6b
7
8
9
10

highly structured fibrillar grid network. There are two types of grid networks, hexagonal (Figures 5.8, 5.9 and 5.10) and orthogonal (Figure 5.11), seemingly irregularly throughout the PM. Average pore sizes are 160 nm and 180 nm respectively with average fibril strands of between 50 nm and 60 nm in diameter, the individual microfibrils having a diameter of about 15 nm independent of the type of grid meshwork. It is possible to have a mixture of the two grid systems adjacent and merging with each other (Figure 5.12). Carbon casts of a hexagonal grid sytem showed its distinct fibrillar nature, for example, at the junction of three fibrils (Figure 5.9) or occasionally where a fibril traverses a pore (Figures 5.8 and 5.10).

Within one to two millimeters posterior to the mouth of the oesophageal invagination the nature of the ectoperitrophic surface layer changes from a structured grid system to a granular layer with little apparent structure (i.e. amorphous). There is a distinct transition beween the two kinds of surface: a close network of microfibrils and ground substance appears to be laid down over the grid (Figure 5.13a and 5.13b), the former as a matted fibrillar support for the granular surface layer (see below). Only this granular ectoperitrophic surface can normally be seen posterior to the transition (Figure 5.14).

5.3.2 Effects of polyphenols *in vitro* and *in vivo*.

When the PM was immersed in gallate or tannate solutions,

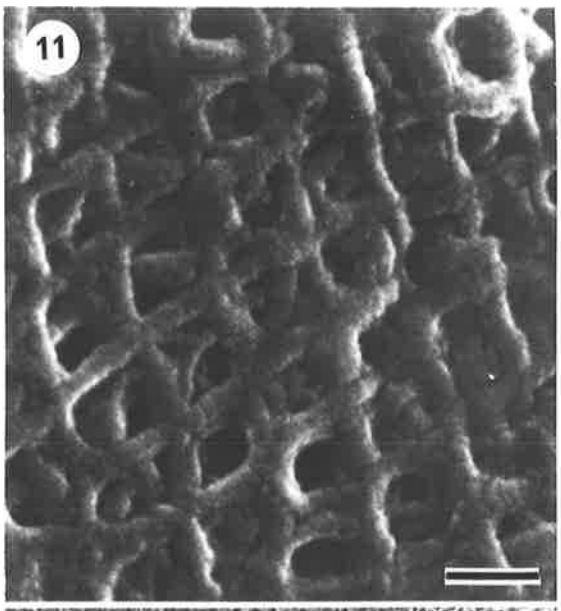
Figure 11 : Ectoperitrophic surface layer showing a largely orthogonal fibrilar orientation. Scale = 0.2 µm.

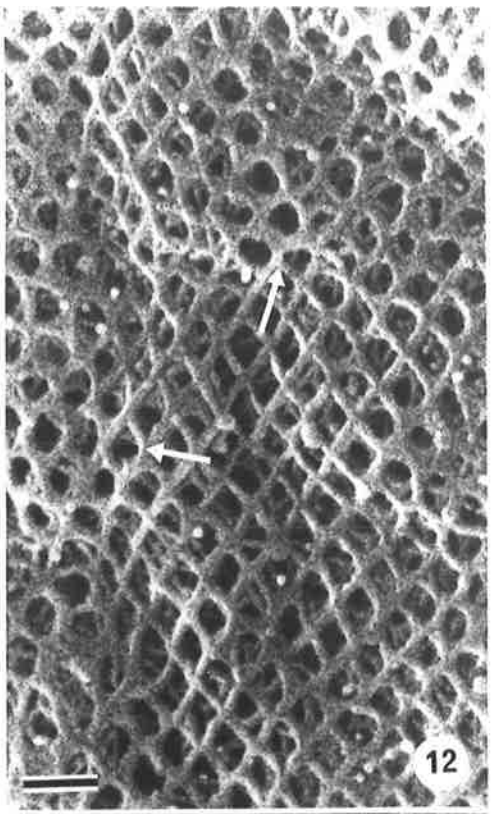
Figure 12 : Ectoperitrophic surface layer showing mixed orthogonal and hexagonal (arrows) fibrilar orientation. Scale = 0.3 µm.

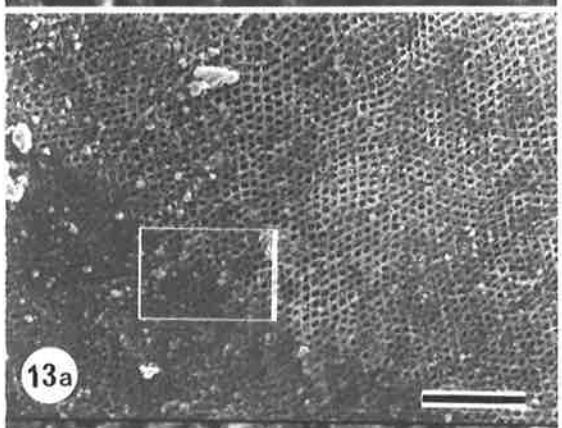
Figure 13a : Deposition of granular ectoperitrophic surface layer about 1 - 2 mm posterior to the mouth of the oesophogeal invagination. Scale = 2 µm.

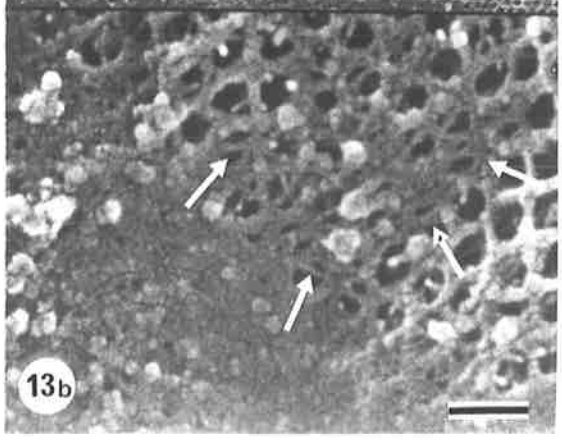
Figure 13b : Enlargement of inset of Figure 13a. Arrows indicate short microfibrils which seems to underly the granular ectoperitrophic surface layer. Scale = 0.3 µm.

Figure 14 : Example of the granular ectoperitrophic surface layer which runs the length of the PM from 2 mm posterior to the mouth of the oesophogeal invagination, into the ileum. Scale = 1 µm.


11


12


13a


13b

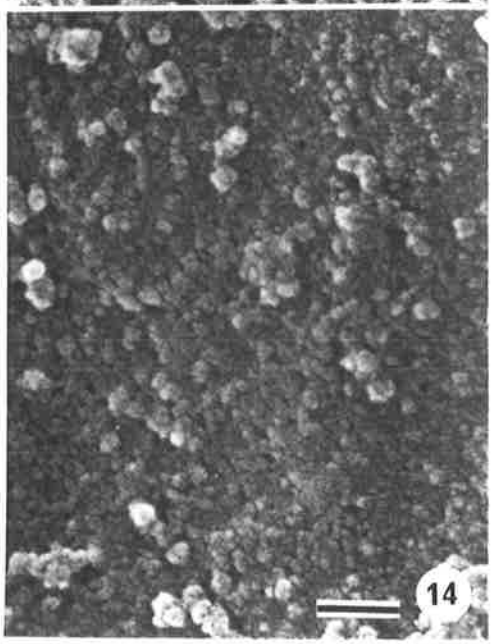

14

TABLE 5.2 : Deposition effects of tannic acid and gallic acid on PM sections *in vitro*.

GALLIC ACID :	pH 3	pH 7	pH 9	pH 11
0.005 M	+	-	?	0
0.025 M	+	-	?	0
0.050 M	++	*,0	?	?
TANNIC ACID :				
0.005 M	++	-	?	0
0.025 M	++	*,0	?	?
0.050 M	+++	*,0	?	?
CONTROLS :	-	-	?	?

+, ++, +++ : increasing effect as described in the text.
- : no effect of test substance seen, i.e. as in control.
? : inconsistent and/or variable effect, unable to differentiate from control.
* : test substance would not dissolve at this concentration and pH.
0 : No trials at this pH or concentration.

consistent results attributable to the effects of the polyphenols alone were obtained only with acidic solutions (Table 5.2). Acidic gallate solutions caused plate-like deposits to be formed (Figure 5.15). Acidic tannate solutions caused flat, ribbon-like deposits with irregularly clumped loci (Figure 5.16), and these deposits were more widespread than the gallate deposits. PMs from Chorticetes terminifera had similar deposits when immersed in an acidic 0.5 M tannate solution (Figure 5.17).

Roaches ingesting gallic acid incorporated in the artificial diet (pH = 6) had an amorphous surface layer of a distinct fibrillar nature, seen clearly at the transition between the grid and amorphous ectoperitrophic surface layers (Figures 5.18a and b) and also posterior to this (Figure 5.19). Under the same conditions of pH and concentration, tannic acid had a more pronounced effect than gallic acid with the amorphous surface layer no longer appearing granular but with a distinct matted fibrillar appearance (Figures 5.20 and 5.21). Also, a lower pH did not seem to enhance this effect, and at lower concentrations the effect was not as pronounced (Table 5.3).

5.3.3 Morphology of the PM in Pa. cribrata.

The granular ectoperitrophic surface layer in Pa. cribrata was similar to that of P. americana's, although much

Figure 15 : PM sections treated *in vitro* with 0.005M gallic acid (pH = 3) showing plate -like deposits on a granular ectoperitrophic surface layer. Scale = 2 µm.

Figure 16 : PM treated with 0.05M tannic acid *in vitro* (pH = 3) showing ribbon-like deposits on a grid ectoperitrophic surface layer. Scale = 0.5 µm.

Figure 17 : Similar deposits to those shown in Figure 16 but on the grid surface layer of a PM from *Chorticetes terminifera*. Scale = 2 µm.

Figures 18 – 19 : Modified granular ectoperitrophic surface layer of the PM from roaches fed 0.5% gallic acid in an artificial diet containing only carbohydrate and silica.

Figure 18a : Deposition of granular ectoperitrophic surface layer 1-2 mm posterior to the mouth of the oesophogeal invagination. Scale = 2 µm.

Figure 18b : Enlargement of inset of Figure 18a. Arrows indicate short microfibrils which seems to underly the granular ectoperitrophic surface layer. Scale = 0.2 µm.

Figure 19a: Granular ectoperitrophic surface layer. Sacle = 2 µm.

Figure 19b : Enlargment of inset in 19a showing modification of the granular ectoperitrophic surface layer. Arrows indicate the more prominent fibrilar nature of this layer. Scale = 0.2 µm.

Figures 20 – 21 : Modified granular ectoperitrophic surface layer of the PM from roaches fed 0.5% tannic acid in an artificial diet containing only carbohydrate and silica.

Figure 20 : Matted fibrilar ectoperitrophic layer. Scale = 0.4 µm.

Figure 21 : Cross-section of a length of PM 10 mm anterior to the ileum-midgut junction, including the endoperitrophic (En) and ectoperitrophic (Ec) space and matted fibrilar ectoperitrophic surface layer (indicated by arrows). Scale = 0.05 µm.

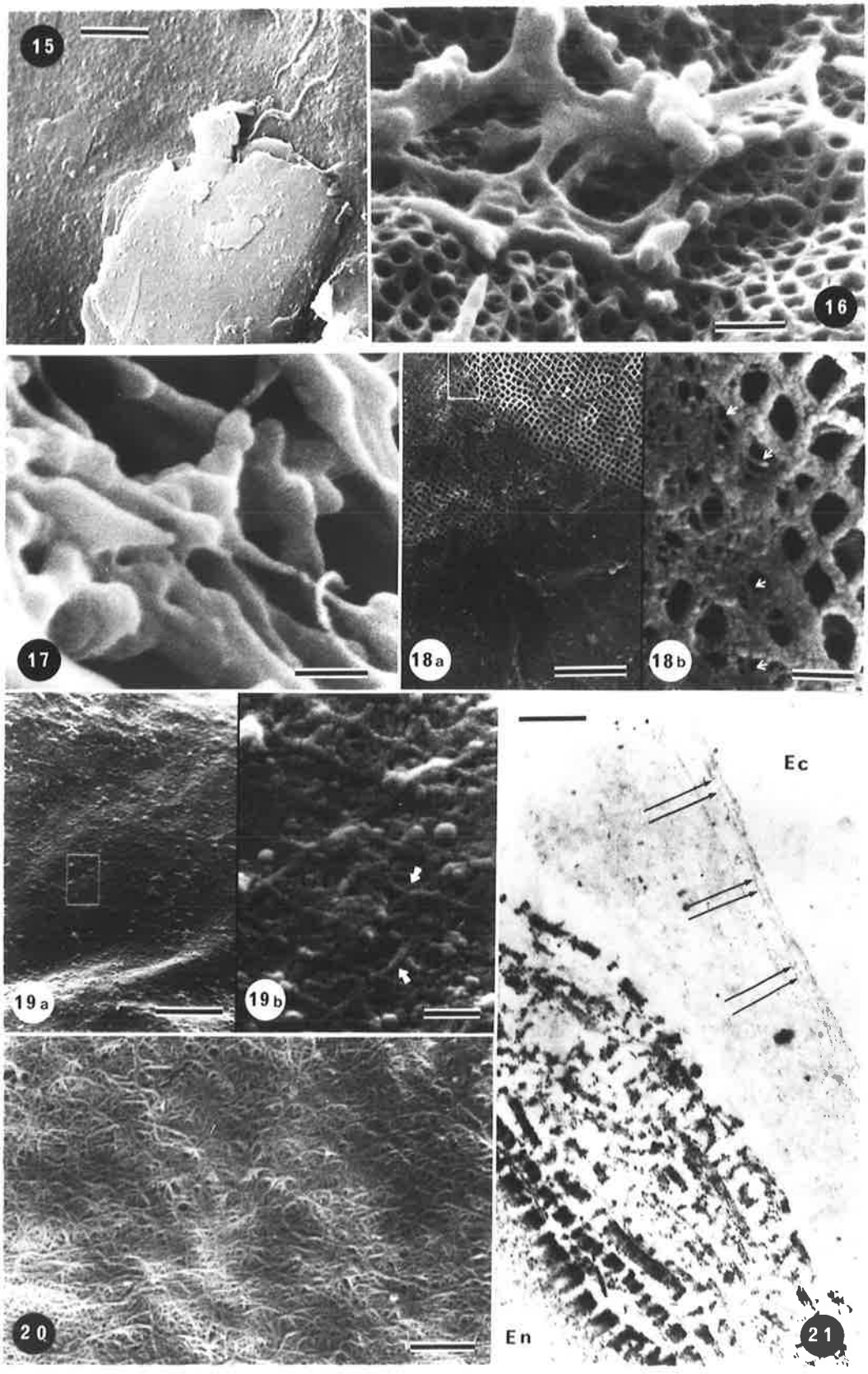

15
16
17
18a
18b
19a
19b
20
Ec
En
21

TABLE 5.3 : Degree of effect of tannic acid and gallic acid on the ectoperitrophic surface layer *in vivo*.

	CONCENTRATION (% of dry weight)		
TANNIC ACID :	0.05%	0.1%	0.5%
(pH 6)	++	++	+++
(pH 3)	0	0	+++
GALLIC ACID :			
(pH 6)	0	0	+

+, ++, +++ : increasing effect as described in the text.
0 : No trials at this pH or concentration.

more prone to colonisation by micro-organisms, e.g. bacteria (Figure 5.22) and fungi (Figure 5.23), probably due to the visible disintegration of the membranes in the posterior midgut. It also had a similar matted fibrillar appearance when an adult female of Pa. cribrata was fed artificial diet containing 0.5% tannic acid (Figure 5.24).

5.3.4 **Fracturography of the PM.**

Fracturography of the anterior secton of the PM shows plastic deformation (Figure 5.25) while posterior sections of the PM have brittle breaks (Figure 5.26). Fractures of the PM (after critical point drying) reveal the variform lamellate nature of the PM with the granular ectoperitrophic surface layer overlying a matted fibrillar layer and grid layers (Figure 5.27).

5.4 **Discussion.**

Mercer and Day (1952) described the PM of P. americana as a complex structure with a characteristic fibrillar network. Closely associated with the fibrillar network were other, unorganised fibrils embedded in an amorphous ground substance, and the components were assumed to consist of chitin and protein respectively. The fibrillar network had hexagonal symmetry; the individual microfibrils forming the fibril strands having a diameter of about 10 nm, and a strand to

Figures 22 - 24 : Ectoperitrophic surface layer from Panesthia cribrata.

Figure 22 : Bacterium apparently dissolving the granular ectoperitrophic layer to reveal the underlying grid layer. Scale = 0.3 µm.

Figure 23 : Fungal hyphae apparently penetrating the granular ectoperitrophic surface layer. Scale = 0.5 µm.

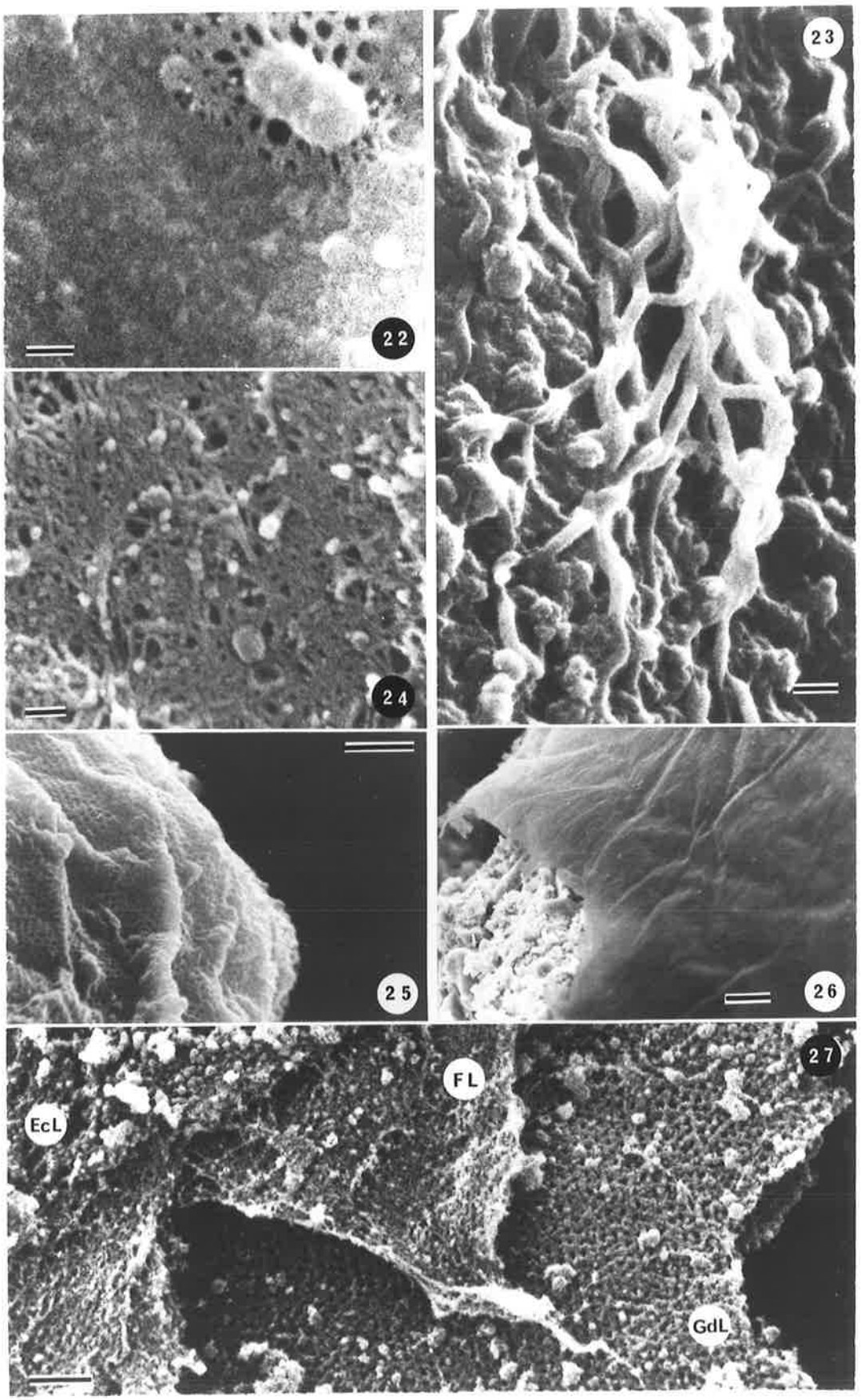
Figure 24 : Modified ectoperitrophic surface layer of the PM from cockroaches fed 0.5% tannic acid in an artificial diet containing only carbohydrate and silica showing a very open matted fibrillar nature. Scale = 0.1 µm.

Figure 25 - 26 : PMs subjected to fracturing with forceps.

Figure 25 : PM section from the anterior midgut showing plastic deformation. Scale = 1 µm.

Figure 26 : PM section from the middle of the midgut showing a more brittle failure. Scale = 0.04 mm.

Figure 27 : Fracture of PM (after critical point drying) showing granular ectoperitrophic surface layer (EcL) with underlying matted fibrillar layer (FL) and grid layer (GL). Scale = 1 µm.


22
23
24
25
26
27
EcL
FL
GdL

strand spacing of between 150 and 200 nm. In this study, individual microfibrils had an average diameter of 15 nm and pore diameters for hexagonal and orthogonal grid meshworks of 160 nm or 180 nm respectively. Due to the similarity of this spacing with the center to center spacing of microvilli, Mercer and Day (1952) suggested that the PM is formed by the secretion of PM precursors into gaps between the microvilli which then acted as a template, thus giving rise to the characteristic hexagonal meshwork. Peters (1976) suggests this is possible for both hexagonal and orthogonal fibrillar networks and that random or feltlike networks of microfibres are formed when polymerisation is slow relative to the rate of production and movement of the PM as a whole. Richards and Richards (1977), however, note that differentiation of microfibrils from a granular layer into an aggregation of larger microfibres can be the result of postsecretion phenomena, even when the mixture is far removed from cell surfaces; the transition can even be seen within relatively short distances within a single PM. Also, meshworks can be formed in unorganised PMs after spatial separation from the midgut epithelium (Richards and Richards, 1971).

Observations made on the ectoperitrophic surface layer in the present study, on grid layers seen in fractures with SEM, and on grid layers rotated slightly in TEM sections, have revealed all three types of microfibrillar orientation; that is, random, hexagonal and orthogonal networks of microfibres.

Although Richards and Richards (1977) suggest there is no significant functional differentiation between the various orientations of microfibres, it may be important to stress here the apparently close association of the randomly orientated microfibres and the granular ectoperitrophic surface layer. Both appeared to be deposited together on the ectoperitrophic surface layer, with the microfibrils arranged randomly but apparently closely associated with the underlying grid system. The granular substance filled any interstices caused by the random microfibrillar arrangement thus giving an overall amorphous appearance. The presence of the granular layer may correspond to the aminopeptidases that Peters and Kalnins (1985) observed immobilised in thin layers on the epithelium side of the PM and to a much lesser extent, right through the PM. The randomly arranged microfibres in P. americana may thus act as a support for aminopeptidases (Peters and Kalnins, 1985) and perhaps other enzymes (see Peters and Kalnins, 1985).

An alternative interpretation of the granular layer and its closely associated, randomly orientated microfibrils is that they could be the initial stage of grid formation with the underlying grid system acting as a template; thus the granular layer, matted fibrillar layer and structured grid systems would be sequential stages of formation. This would imply that the grid system should be the net result at some time after initial secretion of the PM. For reasons explained below, this did not

appear to be the case (although admittedly changes in PM surface morphology were not closely studied over a long period). Nevertheless, it is interesting to speculate on a possible role in grid formation of the aminopeptidases reported by Peters and Kalnins if it were so. Conceivably, they could act to form and arrange the orientated grid systems by altering the conformation of associated proteins, thus also causing the formation of microfibrils (which may explain their sparse distribution right through the PM as seen by Peters and Kalnins, 1985). Perhaps this could be true even if the microfibrils did not eventually contribute to an orientated grid system and the aminopeptidases played a dual role; i.e. in formation of the randomly orientated microfibrillar layer(s) and then as immobilised enzymes for peptide digestion in the ectoperitrophic space.

Changes in the ectoperitrophic surface layer around the oesophageal invagination made it seem likely that the PM is in a dynamic state of production there, i.e. the microfibrils appear to be orientating themselves into a grid meshwork, seemingly while separated from the midgut epithelium. This apparent disorganisation at its anterior end also reflects the fluidity of the PM at this point of the midgut, which Richards and Richards (1977) suggests is necessary for the orientation of microfibrils into grid meshworks. At the same time, the probable state of continuous production of PM at the anterior end of the midgut seems to argue against the possibility that

the granular ectoperitrophic surface layer posterior to the vicinity of the oesophageal invagination is a precursor to the more organised grid system. The granular ectoperitrophic surface layer could perhaps be a detached suface coat of the midgut epithelium but this also seems unlikely since it was present on all PMs including those that had been subjected to repeated handling and washing after removal from the gut.

The ingested tannic acid (and gallic acid to a lesser extent) appeared to prevent the deposition of the granular layer but not the randomly orientated fibrillar network that was always found underlying the granular layer when appropiate observations were made. This argues that the two layers differ in composition. Alternatively, it might seem possible that the secretion of the two layers are sufficiently separated in time that the secretion of the random fibrillar network never happened to be affected by ingestion of tannic acid. This seems unlikely because, as indicated above, the granular ectoperitrophic surface layer and underlying matted fibrillar system were otherwise so consistently found together. It is clear, however, that tannic acid does have a toxic affect on the midgut epithelium which prevents it secreting components of the granular ectoperitrophic surface layer, one of which at least is presumably the aminopeptidase of Peters and Kalnins (1985).

There appears to be no gross alteration of PM structure

in vivo due to tannic or gallic acid deposition such as seen *in vitro*. This may be because of a less saline, or otherwise different ionic composition of the midgut lumen, or because the midgut is neutrally buffered (Bignell, 1982). Martin *et al.* (1985) found tannic acid and pin oak tannins precipitate large amounts of a leaf protein over a wide pH range and in the presence of several cations but again, *in vitro*.

Because of the observable differences that occur in the ectoperitophic surface layer, it was necessary in the experimental determination of possible effects of ingested polyphenols (in a standard diet) on the permeability of the PM, to consider separately the various sections of the PM from different parts of the midgut. The results of such determinations are dealt with in Chapter 6.

CHAPTER 6 : Permeability of the PM *in vitro*.

6.1 Introduction.

Richards and Richards (1977) in their review of the PM in insects found that there was little significant literature on permeability but that it is obvious that digestive enzymes and products of digestion pass through the PM. Recent investigations by Terra and co-workers however, have shown that the PM in insect species of diverse feeding habit and phylogeny partition products of digestion and enzymes such that enzymes involved in the final stages of digestion (e.g. aminopeptidase) are found not to be permeable through the PM (Terra and Ferreira, 1983; Santos *et al*., 1983; Espinoza-Fuentes *et al*, 1984). The PM of *S. gregaria* also appears to be impermeable to some dyes and tannins (Bernays *et al*., 1980; Bernays, 1981b). The above suggests there is perhaps another process by which hydrolytic enzymes are mixed with the ingesta. Zhuzikov (1964) showed a polarity in movement of amylase in the PM of *Aedes aegyptii*; the enzyme appeared to move only from the ectoperitrophic space to the endoperitrophic space across the intact PM, although individual layers appeared to be permeable in both directions. He suggested that the colloidal liquid between the layers must also play a role. Although extrapolation of *in vitro* experiments like Zhuzikov's to the living system might not be valid, when labelled dextrans were fed to larval *A. aegyptii* only compounds with a molecular

weight less than 2,400 Daltons (Einstein-Stokes radius of 1.3 nm - c.f. sucrose of 2 nm and amino acids of 1-4 nm) were permeable (Peters and Wiese, 1986). It is obvious that the PM is a microfilter (i.e. separates particles from solvent) in most species, for example the PM of the Douglas fir tussock moth is permeable to molecules and particulates up to 300-800 nm (Adang and Spence, 1983); but the PM in some species also appears to act as an ultraflter that separates molecules, particularly macromolecules, from solvent thereby effectively concentrating the solute. Further, the separation may be selective or may have a polarity, and these properties may not be confined to just one or two species.

PMs may have other properties, one of which is the prevention of tannin reaching the midgut epithelium (Bernays, 1978, 1981a,b, 1982; Bernays and Chamberlain, 1980; Bernays et al., 1980) due either to the adsorption of tannin (Bernays and Chamberlain, 1980) or some other property relating to tannin penetrability through the PM, e.g. thickness of the PM (Bernays, 1982). The interaction of tannin with the PM is of interest in this study since tannic acid had an affect on the ectoperitrophic surface layer in P. americana and Panesthia cribrata (Chapter 5). Dietary tannic acid had no apparent affect on the intervening grid layers but caused the layer furthest from the ingesta to become more open, perhaps altering the permeability of the PM.

Considerable differences in permeability of PMs are probably found because of inherent differences between insect species and the techniques employed will also greatly influence results (e.g., Zhuzikov, 1970 c.f. Zimmerman and Mehlan, 1976). In this study, a perfusion technique (modified from Zimmerman and Mehlan, 1976) was employed to measure water and dye transport through the PM of P. americana. PMs from cockroaches fed for two hours or three days on diet containing no or 0.5% tannic acid were tested for their hydraulic conductivity and permeability to four dyes. Dyes (including one albumen-dye conjugate) had a size range of 300 - 70,000 Daltons. The effect of NaCl on the permeability of the PM was also investigated.

When the membrane was bathed in water, the low molecular weight dyes themselves caused an increase in the hydraulic conductivity of the PM. Ingested tannic acid had little effect on either water or dye transport. Alcian blue, a large colloidal particle (ca. 1300), appeared to be retained by the PM over time. There was definite retardation of azolbumin (ca. 70,000 Daltons) flux through the membrane associated with negligible water flow. In saline, however, water flow increased when saline was on either side of the membrane. Saline also caused an increase in azoalbumin flux due to an associated increase in water flow.

6.2 **Materials and Methods.**

Techniques are described in this section for measuring the hydraulic conductivity (Lp) of PM's, flux of dyes through the PM (F), and the degree of resistance of the PM (%R) to dyes in cockroaches fed on diets with or without tannic acid.

6.2.1 **Insect Treatments.**

For comparison of the effects of ingested tannic acid on hydraulic conductivity, F, and %R for PM's, adult female cockroaches (used because of their larger diameter PM) were synchronised in feeding as in section 2.1 and fed artificial diet with or without 0.5% tannic acid (section 3.2.1). Groups of females were allowed access to the diet for two hours or for three days. It was known that tannic acid in the longer feeding period affected the PM by changing the ectoperitrophic surface layer (see Chapter 5).

6.2.2 **PM Treatments.**

For comparison of hydraulic conductivity, F, and %R on parts of the PM posterior to the mouth of the oesophageal invagination, sections 3-4 mm long were taken from each third of the PM, thus giving sections from the anterior, middle, and posterior of the PM. The bulk of the food was removed from PM sections by piercing the lumen with a fine needle and breaking

up the compacted ingesta.

6.2.3 **Dye Solutions.**

Five dyes, Azure B (Gurr), Fluoroscein (Gurr), Trypan blue (Gurr), Alcian blue (Gurr) and Azoalbumin (Sigma), with formula weights of 357, 367, 961, ca. 1300, ca. 70,000 Daltons respectively, were used to test the permeability of the PM. Concentrations were chosen so that the filtrate could be assayed routinely without dilution. Solutions were 1.4 x 10-2 mM Azure A, 2.3 x 10-2 mM Trypan blue, 3.1 x 10-2 mM Alcian blue, and 6.1 x 10-3% Azoalbumin.

6.2.4 **Measurement of hydraulic conductivity (Lp).**

Hydraulic conductivity can be calculated from the volume flow, Jv, ($cm^3/cm^2.sec$) across the PM under a hydrostatic pressure, ΔP, (House, 1974):

$$Jv = Lp. \Delta P \qquad (1).$$

A simple blind perfusion technique was developed where Jv could be measured directly from the actual water flow, Jw, since:

$$Jv = Jw/SA/t \qquad (2);$$

where SA is the surface area through which the flow (Jw) occurred over time t. Rearranging (1) and (2) gives:

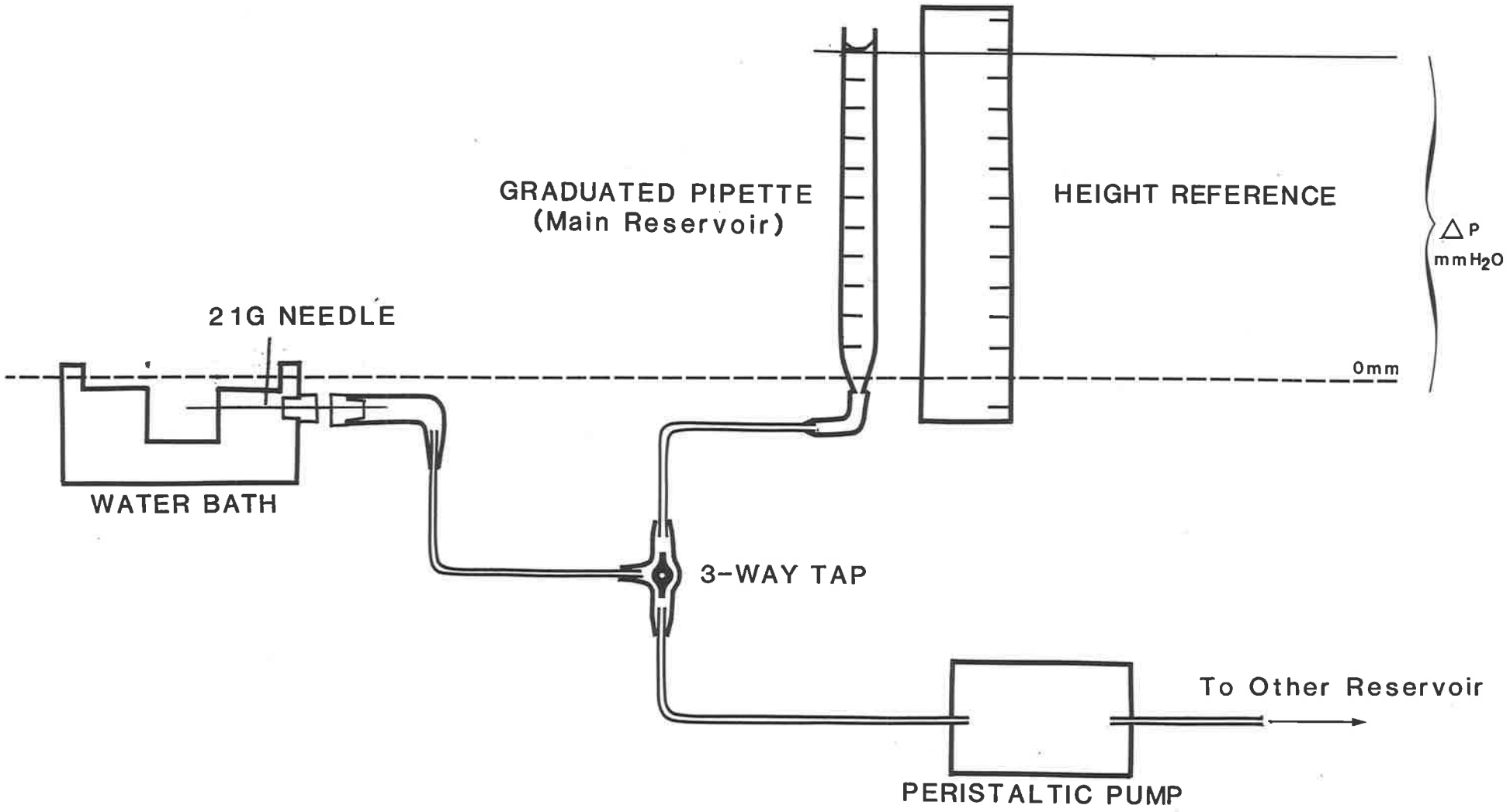
$$Lp = Jw/SA/t/\Delta P \qquad (3).$$

Filtration techniques have potential sources of error due to the small surface areas normally employed (see Zimmerman and Mehlan, 1976, cf. Zhuzikov, 1970). The apparatus consisted of a perspex block, 35 mm square and 16 mm high, with a central well 10 mm deep and 13 mm in diameter into which a 21G needle was inserted, via a rubber septum (Figure 6.1). The area around the well (30 mm in diameter) was slightly inset by 3 mm. Externally, the other end of the needle was attached via a rubber septum and a three-way tap to a 1 ml graduated pipette. All interconnexions were by short lengths of polyethylene tubing. A desired hydrostatic pressure was achieved by regulating the head of water in the pipette with respect to the height reference scale (Figure 6.1) adding or withdrawing solution by means of a Pharmacia P-3 peristaltic pump (connected with the pipette by the three-way tap).

Sections of PM 3-4 mm in length were first tied loosely onto the needle with nylon thread and flushed with the test fluid until all food was washed from the lumen. The section was then untied and removed from the well with a plastic spatula. The well was cleaned and filled with new bathing fluid. Strips of teflon tape approximately 0.5 mm in width were used firstly

FIGURE 6.1

Apparatus used to measure hydraulic conductivity and dye flux of PM sections *in vitro*.


GRADUATED PIPETTE
(Main Reservoir)
HEIGHT REFERENCE
ΔP
mmH2O
21G NEEDLE
0mm
WATER BATH
3-WAY TAP
To Other Reservoir
PERISTALTIC PUMP

to tie off one end of the PM and create a bag, secondly, to tie the bag onto the needle (see Figure 6.2). There were neglible differences in length measurements of membranes tied or untied so surface area (SA) of the PM was assumed to approximate that of an open-ended cylinder:

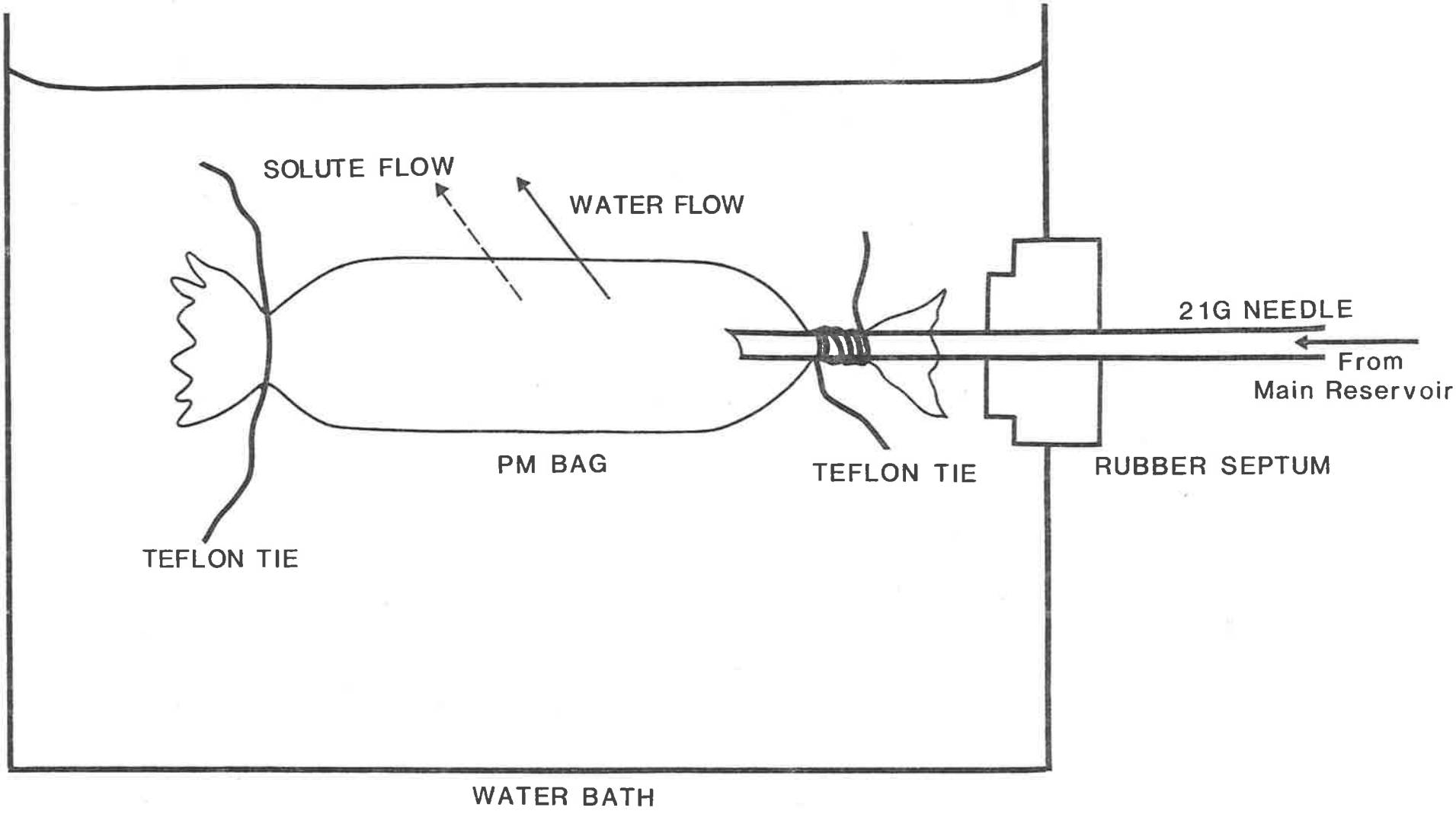
$$SA = 2.r.\pi.l \qquad (4),$$

where 2.r is diameter and l length of the PM. Double distilled water was used in the reservoir and water bath. Water flow, Jw, was measured as the drop in the head of water in the pipette and read directly from the graduated pipette (0.01 ml divisions) and the time taken recorded. Around 0.02 ml over three minutes was common for the determination of hydraulic conductivity. Although water flow caused a corresponding drop in hydrostatic pressure, the proportionate change and effect on hydraulic conductivity appeared neglible as long as the drop was less than 5 mm. Hydraulic conductivity results for PM sections were accepted only if they reached a maximum value that remained consistent for two or more hydrostatic pressures at 50 mm intervals (see section 6.3.1).

Bath contents were gently agitated by drawing bathing solution into a pipette then expelling it back into the water bath. All manipulations and measurements were carried out in a room kept at 20 $\pm$ 1 ^{o}C.

FIGURE 6.2

PM section tied onto needle in a water bath as a blind bag.


SOLUTE FLOW
WATER FLOW
21G NEEDLE
From
Main Reservoir
PM BAG
TEFLON TIE
RUBBER SEPTUM
TEFLON TIE
WATER BATH

6.2.5 Measurement of Dye Flux and calculation of % Resistance.

Dye flux, or the net movement of dye across the PM due to hydrostatic pressure, was measured as number of moles per second, after accounting for differences between sections in water flow, surface area, and hydrostatic pressure. Hydraulic conductivity was also determined when dye flux was determined. PM sections were perfused as in 6.2.4 except that dye solutions replaced double distilled water in the reservoirs; double distilled water was the initial bathing solution. Aliquots of 0.08 ml of the stirred bathing solution, before and after dye flow, were determined colorimetrically on a Varian DMS 90 double beam spectrophotometer. Wavelength maxima for Azure B, Trypan blue, Alcian blue and Azoalbumin are 615, 590, 613, and 335 nm respectively. The observed number of moles, n_o, crossing the PM could then be calculated from the increase in dye concentration of the bathing solution.

Sampling without replacement caused a decrease in the level of the water bath but this was never allowed to exceed 5 mm. Double distilled water was added when necessary to make up to the level. Another error was that the initial volume of the bathing solution (approximately 5.0 ml) was changed slightly as liquids perfused the membrane, but the change in volume (0.03 ml) for changes in hydrostatic pressure less than 5 mm of water appeared neglible and the treatment gave consistent differences

between treatments (with different dyes).

Because of differences in dye concentration used to perfuse the membrane, flux between different dyes is not strictly comparable. But the per centage resistance could be estimated for each of the dyes from the observed number of moles, n_o, and the calculated number of moles, n_c, which is the maximum number of moles that would cross the membrane per unit flow of solution if the dye concentration remained unaltered through the membrane. It was assumed that the concentration within the lumen of the PM was equal to that in the reservior. %R can then be calculated by:

$$\%R = (n_o - n_c / n_c) \times 100 \qquad (5).$$

As a ratio, %R is similar to the reflexion coefficient (as described in House, 1974) which is derived from equations assuming a flow due to a differential in osmotic pressure rather than one caused by hydrostatic pressure. %R is here proposed to describe that per centage of solute that is retarded by a membrane when flow across the membrane is due to a hydrostatic pressure.

6.2.6 **Determination of hydraulic conductivity for Azoalbumin, <u>in situ</u>.**

When flux and hydraulic conductivity were determined for

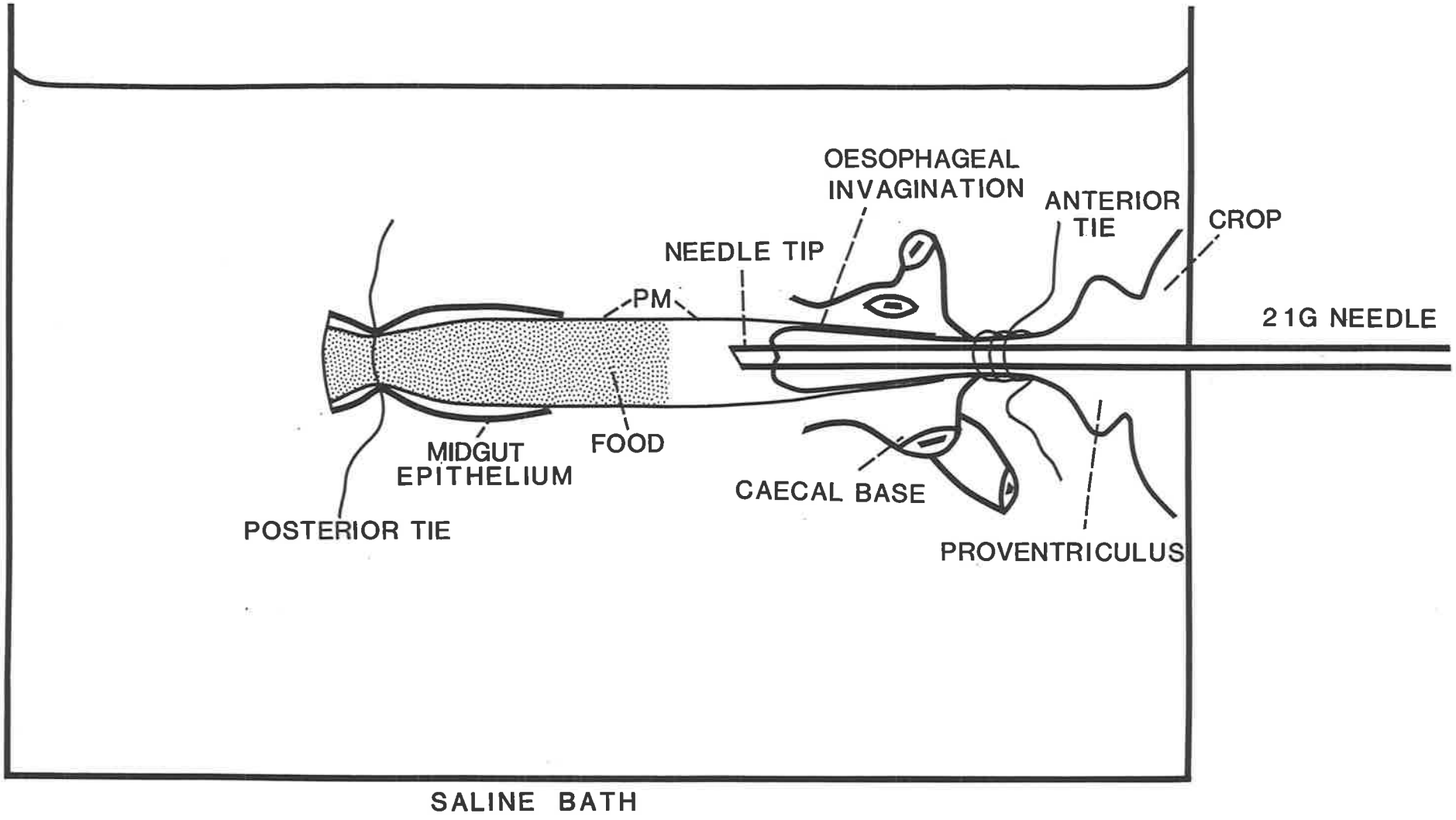
Azoalbumin, it was apparent that Azoalbumin interfered with water movement since hydraulic conductivity was also markedly reduced. Enzymes are considered to cross the PM *in vivo* and hence this result was clearly problematical. There were other ways that enzymes and large proteins could cross the PM (see 6.4) but of interest here was the possibility that the PM overlying the oesophageal invagination was much more permeable or discontinuous than the rest, and was the site for enzyme and/or protein transport. The PM overlying the oesophageal invagination could not be handled as above (section 6.2.4) due to the fragility of the PM and its apparent fluidity here. An attempt was made therefore, to assess the permeability of the PM in this region *in situ*.

The gut was prepared basically as in section 2.2. It was found that when the preparation was bathed in water, the PM soon sloughed off the oesophageal invagination, whereas saline kept the preparation intact (perhaps by preserving the continuity of the origins of the PM with the secretory cells, although this possibility was not investigated). All operations were therefore carried out in a 0.09% NaCl (w/v) solution. The crop was opened to expose the proventriculus and excess tissue removed. The proventriculus was washed clear of foodstuff with a flow of saline from a pipette to avoid contamination and blockage of the needle on its subsequent insertion (see below). A nylon ligature was placed around the midgut about one centimetre posterior to the oesophageal invagination and pulled

just tight enough to arrest the PM through the midgut epithelium (see position of posterior tie in Figure 6.3). The gut posterior to the ligature was discarded and the rest, from proventriculus to ligature, was transferred to the well. The needle was forced through the part of the proventriculus which leads to the midgut, but was not allowed to pass the mouth of the oesophageal invagination. A small puncture was then made anterior to the ligature, penetrating through the midgut epithelium to the midgut lumen but not rupturing the PM. The hole thus made was gradually enlarged as gut lumen pressure equalised with bath hydrostatic pressure. Caeca were then cut close at their base and discarded allowing a passage for water and solute flow to and from gut lumen and saline bath. The midgut epithelium was then carefully trimmed away with a microscalpel to expose the section of PM immediately anterior to the tip of the oesophageal invagination; this part of the gut normally contained no food (as in Figure 6.3). The end of the needle could then be positioned easily so that it protruded just past the mouth of the oesophageal invagination into the PM lumen. To secure the gut preparation onto the needle, a teflon ligature was placed just below the proventriculus (as in Figure 6.3). The surface area of the PM was estimated as before, except the length was taken to be from the base of the caeca to the start of the compacted mass of ingesta; it was assumed that there would be little penetration of water and dye into the bulk of the ingesta over short time intervals (1-3 mins).

FIGURE 6.3

Anterior midgut section tied onto needle for *in situ* determination of hydraulic conductivity.


OESOPHAGEAL
INVAGINATION
ANTERIOR
TIE
CROP
NEEDLE TIP
PM
21G NEEDLE
MIDGUT
EPITHELIUM
FOOD
CAECAL BASE
POSTERIOR TIE
PROVENTRICULUS
SALINE BATH

6.2.7 **Saline Treatments.**

Some determinations of hydraulic conductivity of excised PM sections were made with all combinations of either saline or water on either side of the PM bag as in 6.2.4. The saline solution was 0.09% NaCl (w/v) as used in 6.2.6. Azoalbumin solution was perfused through PM sections bathed in saline; hydraulic conductivity, dye flux and %R were determined.

6.3 **Results.**

Results were analysed with Genstat V (Copyright, Rothamstead Experimental Station) using analysis of variance. No difference was found between hydraulic conductivities for different PM sections from different parts of the midgut nor from cockroaches fed either for two hours or for three days. The results for these treatments were therefore combined.

6.3.1 **Validity of Measurements.**

Increasing hydrostatic pressure will cause an increase in hydraulic conductivity until an upper limit is reached (Zimmerman and Mehlan, 1976), when changing the hydrostatic pressure should cause no further changes in hydraulic conductivity. This level appeared to be reached in most cases at about 150 mm H_2O. If leaks were present, then hydraulic conductivity increased markedly with increasing pressure. To

check for any suspected leaks, PM sections, after hydraulic conductivity determination, were subjected to water flow at high pressures (> 250mm H_2O) while the well was flooded with 0.1% Toluidene blue (BDH). Any abnormal water flow (from a leak) then showed up as a pale jet that contrasted with the Toluidene blue. Further evidence of the validity of the method is given by comparisons of hydraulic conductivity determinations from the same section of PM but after shortening its length, thus reducing its surface area. Indeed, if the membrane was suspected of leaking where it was tied onto the needle, then it was retied with a subsequent decrease in its length. Hydraulic conductivity determinations were considered valid if there was less than a 1% error for a 10% decrease in surface area.

The maximum error calculated for the apparatus was 10% for hydraulic conductivity and 15% for flux determination. Error was higher for flux determinations because of the added dependence of the calculation on the total volume of the well and sampling without replacement. Errors in dye flux and %Resistance due to lack of stirring the solutions in the PM bag could not be estimated and could have become significant in some circumstances (e.g., the effect seen with Alcian blue - see below). Nevertheless, the results did appear consistent and did show marked differences between some treatments.

6.3.2 Effect of Tannic Acid on Permeability Parameters of PM Sections.

To determine the effect of ingested tannic acid, permeability parameters for sections of PM from cockroaches fed for five days with or without 0.5% tannic acid incorporated into a standard diet (see section 6.2.1) were compared. Table 6.1 shows there is little apparent effect of ingested tannic acid on the subsequent permeability of PM sections measured *in vitro*, in relation to hydraulic conductivity, dye flux or %Resistance. There are two anomolous results where there was a marked difference for Trypan blue in hydraulic conductivity but not dye flux, and for Alcian blue in %Resistance but not dye flux.

The results given for dye flux of Alcian blue are the maximum values observed since there was a marked decrease in water flow over time. There was an apparent complexing of dye in the lumen of the PM bag forming a layer of particles on the PM's endoperitrophic surface layer, increasingly blocking the pores over time. Thus the difference in %R between the control and tannic acid treatments with respect to Alcian blue although not significant, may be an artifact due to blocking of the pores.

TABLE 6.1 : Hydraulic conductivity (Lp), dye flux (F), and percentage resistance (%R) of PM sections *in vitro*.

DIET	SOLUTIONS Reservoir: water Bath : water	AZURE A water	TRYPAN BL water	ALCIAN BL* water	AZOALBUMIN water	 saline
WITHOUT TANNIC ACID	Lp** 0.091[a]	0.136[b]	0.109[c]	0.115[c]	0.006[d]	0.060[e]
	$Fx10^{-6}$# -	1.49	3.71	3.03	1.07	8.05
	%R** -	0.3[a]	3.5[a]	17.2[b]	92.2[c]	88.9[c]
WITH TANNIC ACID	Lp** 0.081[a]	0.134[b]	0.067[c##]	0.120[b]	0.005[d]	0.062[c]
	$Fx10^{-6}$# -	1.64	3.83	4.06	0.83	10.58
	%R** -	-0.1[a]	-1.2[a]	-0.3[a]	95.8[b]	85.2[b]

* maximum value only

\# no signifcant difference between PM sections from cockroaches fed diet with or without tannic acid; not comparable between dyes (see text).

** no signifcant difference between PM sections from cockroaches fed diet with or without tannic acid except as indicated by "##".

\## exception to the above: Lp values for Trypan blue differed between PM sections from cockroaches fed diet with or without tannic acid ($P < 0.05$).

6.3.3 Comparisons of Permeability Parameters for Water, Azure B, Trypan blue, and Alcian blue.

The three non-conjugated dyes, Azure B, Trypan blue, and Alcian blue caused an increase in hydraulic conductivity, and the PM appeared to offer little resistance to them. In the presence of tannic acid, %R appeared very slightly negative (Table 6.1). Values for resistance may be somewhat underestimated, however, due to an inherent error of assuming that luminal concentration reflected the reservoir concentration; whereas unstirred layers next to the luminal surface of the PM bag may effectively have higher concentrations than the reservoir, thus increasing the concentration gradient somewhat and therefore the rate of diffusion of dye through the membrane.

Of the three dyes above, the smallest, Azure A, caused the highest hydraulic conductivity. Clearly these three dyes do not compete with the movement of water molecules but enhance it, perhaps by distortion of either the fibrillar structure or, more probably, the proteinaceous matrix. Azoalbumin on the other hand, severely curtailed flow of molecules through the membrane.

6.3.4 Comparisons of Permeability Parameters for Water and Azoalbumin and effects of saline bathing solution.

When the bathing solution was water the hydraulic conductivity was substantially suppressed by Azoalbumin. If enzymes cross the PM *in vivo*, e.g. to be regurgitated into the crop, then from this result, it would appear that the enzymes produced by the midgut enter the gut lumen other than through the parts of the PM represented by the sections tested. This would leave the possibilty of passage through the PM where it overlies the oesophageal invagination or by other means, e.g. if caeca lumina were continuous with the PM lumen.

Replicate consistent values of hydraulic conductivity for the *in situ* anterior PM preparations in the presence of Azoalbumin were 0.96, 0.110, 0.064 cm/s.bar. Since the preparation was bathed in saline to prevent the PM sloughing off the oesophageal invagination (see 6.2.6), the comparable value for separated sections of PM was 0.060 cm/s.bar (Table 6.1 - see below). If the caecal lumina were continuous with the PM lumen, however, then hydraulic conductivity should be very large in such preparations since the PM would no longer act as a barrier. Also, with Azoalbumin present, the route that the dye took should have been directly and easily visible; however, the observed route for diffusion of Azoalbumin was across the PM posterior to the oesophageal invagination. The results for the *in situ* PM on the oesophageal invagination therefore imply either that the caecal lumina are not continuous with the PM lumen or that the anterior midgut is adpressed so closely to the PM overlying the oesophageal invagination that liquid flow

between the two is severely impeded.

At the same time, in the presence of Azoalbumin, the hydraulic conductivity of the PM, in situ on the oesophageal invagination and bathed in saline, was considerably higher than the value obtained for separated sections of PM bathed in water (0.006 cm/s.bar). This pointed to a possible dramatic effect of saline itself (compared with water) on the PM. To test the effect of saline on the PM futher, hydraulic conductivities, dye flux and percentage resistance were measured when separated sections of PM were perfused with Azoalbumin solution and bathed in saline; the results are indicated in the last column of Table 6.1. The increased water flow in saline had also resulted in an increase in dye flux. Further, it was established through the experiment that the effect was reversible when the bathing solutions were alternated from water to saline and back again.

The reversibility of the phenomenon appeared to indicate that NaCl altered the permeability of the membrane in some physical way rather than, for example, changing the chemical structure of its constituent protein. To test this possibility further, changes in the hydraulic conductivity of PM sections when transferred to different solutions were determined as described in section 6.2.7. Table 6.2 gives the results for six PM sections, two of which are from cockroaches with tannic acid incorporated in their food. It was apparent that saline on either side of the membrane caused an increase in hydraulic

TABLE 6.2 : Effect of different combinations of saline on hydraulic conductivity (Lp) of some PM sections *in vitro*.

SOLUTIONS IN Pipette/Bath	PM SECTIONS - WITH TANNIC ACID* (replicate values)				PM SECTIONS - NO TANNIC ACID* (replicate values)	
	1	2	3	4	5	6
	Lp (cm / s . bar)					
water/water	0.074	0.105	0.053	0.085	0.087	0.032
water /saline	0.101	0.152	0.070	0.128	0.121	0.048
saline/saline	0.092	0.205	0.075	0.155	-	0.049
saline/water	0.093	0.186	0.069	0.146	-	0.047

* PM sections were from adult female cockroaches fed a standard diet with or without tannic acid as indicated (see text for more details).

conductivity ($P < 0.05\%$) with perhaps a slightly larger increase when saline was on both sides of the membrane (e.g. as in PM sections 2,3,4, and 6). The effect of saline was apparent for PM sections from roaches irrespective of whether they had ingested diet with or without tannic acid.

6.4 **Discussion.**

The mean hydraulic conductivity of PMs from female P. americana fed a standard diet was 0.091 cm/s.bar. When PMs were from cockroaches that had ingested standard diet with 0.5% tannic acid, the hydraulic conductivity was non-significantly different (0.081 cm/s.bar). It thus appears that the outer layers of the PM which are affected by ingested tannic acid as discussed in Chapter 5, at least in vitro, play no important role in regulating or preventing flow of water (or solutes - see below) across it. Supporting evidence is that the very outer layers could be pierced with a fine needle without affecting hydraulic conductivity. Azure A, and to a lesser extent, Trypan Blue and Alcian Blue, increased the hydraulic conductivity.

As Lehane (1976) suggests for the mucopolysaccharide layers of the PM in Stomoxys calcitrans, permeability may be partly dependent on steric effects (which may explain the differences between dyes) and partly on energetic effects

according to Ogston's steric exclusion theory (Ogston, 1958, cited in Lehane, 1976). Thus pH or other ionic changes may influence the PM by causing conformational changes leading to changes in effective pore size and the fixed charge density. Lehane suggests that a change in the latter could cause a disproportionate change in permeability. This could explain the effect of saline which increased hydraulic conductivity by about 150% for water/saline combinations and increased hydraulic conductivity for saline/azoalbumin solution combinations ten-fold with a corresponding increase in dye flux of eight to ten-fold. Clearly, azoalbumin must have decreased water flow in the absence of saline due to very effective blocking of pores by the protein molecule and the effect was almost removed by the presence of saline, presumably due to an effective increase in pore size.

Zimmerman and Mehlan (1976) also showed an affect of various salts on the hydraulic conductivity of Calliphora erythrocephala larval PMs, although they found that NaCl, K_2SO_4, and $CaCl_2$ decreased rather than increased hydraulic conductivity while KCl and KSCN increased hydraulic conductivity. The differences between the two species may simply reflect differences in structure of their PMs, dipteran PMs consisting mainly of colloidal layers, not loose and open grid systems as in the cockroach (Chapter 5). It is also noteworthy that the hydraulic conductivity of larval and adult C. erythrocephala are much lower (0.025 cm/s.bar and 0.014

cm/s.bar respectively) than those of P. americana (see above).

The decrease in hydraulic conductivity due to tannic acid seen with Trypan blue could be due to a chance combination of several factors, perhaps dye shape, size and charge distribution and alteration of charge density by tannic acid. Although Alcian blue has a higher molecular weight than Trypan blue it differs considerably from Trypan blue with respect to all the above factors; thus the lack of difference in hydraulic conductivity with tannic acid may not necessarily cast doubt on the results for Trypan blue.

Lee (1968) considered that the caecal lumina were continuous with the endoperitrophic space, which would readily explain the passage of enzymes into the endoperitrophic space (this despite his showing that PM laminae could often be traced to clefts in the midgut epithelium between caeca and foregut intima). Hydraulic conductivity for PMs when tested in situ on the oesophageal invagination (i.e. with anterior midgut epithelium still covering the PM on the invagination) was similar, however, to the expected value for the PM as a discrete section in vitro.

If enzymes are dependent on crossing the PM, then several factors could influence such transport: size, charge, and shape of the enzymes; the thickness, structure and chemical composition (e.g. possible presence of mucopolysaccharide

colloidal layers), pore size and charge density of the PM; the microenvironment at the site of crossing the PM, including pH and composition and concentration with respect to other solutes (especially perhaps the cations). The above factors could well regulate the movement of enzymes, e.g. by controlling the flux and concentrations of basic cations.

Results suggest that the PM in vivo most likely acts as a microfilter separating particulate matter from solution. Waterhouse (1957) suggested that the PM by retaining solids would increase the efficacy of absorption since fluid movement in the ectoperitrophic space could then be independent of the backward passage of the compacted ingesta. This could effectively create an unstirred layer next to the midgut epithelium and retain solutes in the midgut longer, increasing the time for digestive and absorption processes. Further, the midgut epithelium would not become clogged with particulate debris.

CHAPTER 7 : GENERAL DISCUSSION.

7.1 PM involvement in digestion and assimilation of nutrients.

Dow (1986) gives an overview of feeding and digestion in relation to midgut function for several gut morphologies and feeding habits. For the cockroach, classed as a generalist feeder with a simple gut, he states in summary that food is mixed with enzymes from saliva and midgut, and is digested in the crop. It is then passed gradually into the midgut where solubilised nutrients and other solutes are absorbed in the caeca and more digestion occurs as the food passes back along the midgut into the hindgut (ileum). Dow thought it possible, although not conclusive, that fluid derived from the primary urine was passed forwards in the ectoperitrophic space (see also Bignell, 1982), i.e. creating a countercurrent system (Dow, 1981a,b). It seems to be agreed that the main absorptive area in the midgut is provided by the caeca since ingested dyes are concentrated there, also indicating that the caeca may be the site for absorption of water, ions and other solutes (in Bignell, 1982). Dow (1986) suggests this would probably be due to a basal Na^{+}/K_{+} ion pump with associated passive transport of water and solutes. Undigested material is passed back into the hindgut which, according to Bignell (1982) has a low redox potential that is presumed to reflect microbial activity.

In the present study, there was strong corroboration for the accumulation of solutes from ingesta in the caeca, and again some evidence in favour of a countercurrent flow, but only when adult male cockroaches that were apparently feeding were injected with dyes.

In some insects there may be a partitioning and control of enzyme composition whereby those hydrolases that are involved in the final stages of digestion are restricted to the ectoperitrophic space, either because they are too large to cross the PM or because they are membrane-bound, while those enzymes involved in initial digestion can cross the membrane freely when unbound by substrate. These enzymes when bound with a polymer substrate, however, are confined to the PM lumen and enter the ectoperitrophic space in the posterior region of the midgut only once the substrate has been degraded and is therefore smaller. Solutes and enzymes in the ectoperitrophic space are carried to the anterior region of the midgut by a countercurrent sytem, thereby conserving nutrients and enzymes (Terra et al., 1979; Terra and Ferreira, 1981, 1983; Ferreira et al., 1981; Santos et al., 1983; Espinoza-Fuentes et al., 1984).

Dow (1986) does not account in his model for enzyme movement or PM production although his idea of a countercurrent flow might explain the accumulation of both ingested and injected dyes in the caeca found in the present study. Food is

quite obviously compacted in the PM of previously fasted cockroaches only one day after feeding on a variety of diets (see Chapter 3 and Bignell, 1977); this solid ingesta is seemingly packaged thereby for despatch to the hindgut where it is further processed. Compaction of the ingesta would also seem likely to prevent further entry of enzymes into the bulk of the ingesta, and could well subserve their conservation (see below), for it would appear wasteful for the cockroach to loose active enzymes in this stationary phase where they could well be inactivated and/or lost to the hindgut where microflora would further inactivate and degrade them. The compaction of food in the PM may, to some extent, explain the concentration of dyes in the anterior midgut. These possibilities are further explored below.

As considered in Chapter 6, if activity of digestive enzymes is dependent on their crossing the PM, then several factors could influence this transport: size, charge, and shape of the enzymes; the thickness, structure and chemical composition (e.g. possible presence of mucopolysaccharide colloidal layers), pore size and charge density of the PM; the microenvironment at the site of crossing the PM, including pH and composition and concentration with respect to other solutes (especially perhaps cations). It is possible that these factors could regulate the movement of enzymes, e.g. their transport within the gut could be controlled by the flux and concentrations of ionic species.

Other physical properties of the system that should also be considered include the production and anterior-posteriad movement of the PM in response to feeding (Chapters 2, 3 and 4), and the influence of crop pressure on the system. Also, the fluidity and relative disorganisation of the grid system overlying the oesophageal invagination (Chapters 2 and 4) and the movement and nature of the oesophageal invagination (e.g. Chapter 4 describes microtrichia covering the oesophageal invagination) need to be taken into account. Compaction of food into the PM could also influence the dynamics of solute flow, especially if associated with a countercurrent system as suggested by Dow (1986).

The movement of the PM through the midgut is probably assisted by pressure applied from contractions of the crop (O'Riordan, 1968, cited in Bignell, 1982). Associated with this backwards movement is the synthesis of the PM by cells of the anterior midgut and the polymerisation of chitin-protein complexes into microfibrils in the region of the midgut near the oesophageal invagination. This newly formed PM must then contend with the contractions of the oesophageal invagination and associated movement of the microtrichia. The fluidity (as defined in section 2.3) of the PM would presumably help keep its integrity while allowing flexibility. This is supported by the observation that the PM can undergo an approximate 16% extension under a 1.2 g load for 72 secs (Chapter 2). There is an apparent lack of organisation of the ectoperitrophic surface

layer too (Chapter 5) in this region, possibly facilitating greater permeability.

The microtrichia of the oesophageal invagination appear to be evenly distributed implying that the pressure on the PM would also be evenly distributed, helping to prevent rupture while it is in its initial fluid state. In most general discussions of digestion in the cockroach, the anterior region of the midgut is considered to be a site where enzymes are secreted and presumably must pass through the PM. The flexibilty of the membrane here suggests that, as it stretches immediately beyond the oesophageal invagination there will be a change (i.e. increase) in effective pore size, possibly enabling enzyme flux in this region. It would also appear likely that movement of enzymes across the PM may be facilitated by anteriad movement of water associated with regurgitation of fluid into the crop (Bignell, 1982).

The compaction of food in the PM appears to be the result of crop pressure forcing a slurry of partly digested food into a newly secreted (Chapter 4) membrane which retains the solids but allows the outward passage of solutes through most of the length of the PM. When the cockroach first feeds after fasting, the compaction of ingesta proceeds throughout the length of the midgut, but when the cockroach has been feeding for some time, then compaction of ingested materials occurs in the anterior region only of the midgut, since the posterior section of the

PM is now already full of compacted ingesta (Chapter 3). In the latter situation, therefore, as the slurry of partly digested food enters the anterior region of the PM lumen, solids would be retained and solutes would cross the PM just posterior to the caeca which would also explain the concentration there of ingested dyes, even without recourse to the countercurrent hypothesis, although such a countercurrent may improve retention of solutes in this region; such retention would maximise the absorptive capacity of the larger surface area (due to the caeca) in this region, particularly when food is limited or not ingested (e.g. at some periods within the reproductive cycle of the female roach - Rollo and Gunderman, 1984). Enzymes in solution would also be retained in this region; such localisation here could well lead to conservation of the enzymes and maximize utilization of their activity.

Results suggest that the PM may act as an ultrafilter under some conditions (Chapter 6) and certainly as a microfilter. Further, solutes and perhaps enzymes could remain in the midgut for much longer periods than initial passage of particulate ingesta through the midgut might suggest (see e.g. evidence for the retention of some dyes in Chapter 3). Retention of fluids in the midgut was first suggested by Waterhouse (1957) as another possible effect of the PM. It further maximizes the process of digestion by allowing microbial fermentation of the particulate ingesta passed back to the hindgut without having to wait for the completion of

digestion in the midgut.

Such a division of labour between parts of the midgut as described above, combined with immobilised enzymes on the ectoperitrophic surface layer (Peters and Kalnins, 1985) and other properties of a surface coat, e.g. adsorption of sugars, (Peters et al., 1985), and partitioning of enzymes (Terra and co-workers - see section 5.1) provide potential for a very dynamic and efficient digestion and assimilation system for insects in general.

7.2 **Effects of tannic acid on the PM.**

When 0.5% tannic acid was fed to cockroaches (individuals of both P. americana and Panesthia cribrata) for three days, the ectoperitrophic surface of the PM was seen as an open fibrillar meshwork compared with the amorphous, granular layer normally seen in controls (Chapter 5). Further, there appeared to be an apparent slowing of ingestion of diet and of passage of food back to the hindgut (Chapter 3). By five days after first feeding, the amount of faeces passed out was much lower compared with controls. Whether tannic acid is toxic to the cockroach is difficult to assess since the diets did not also contain all the cockroaches' dietary reqirements, but there was a clear effect of tannic acid on the ectoperitrophic surface layer which does suggest a toxic effect if aminopeptidases are immobilised in the amorphous surface layer (Chapter 5). The

potential of tannic acid for disrupting the digestive system as described above needs further study, but it is apparent that, in non-adapted species, low concentrations may have pronounced effects (Bernays, 1981a). Since the PM is one of the structures tannin has been reported to affect, it may well play a role in determining the dietary effects of tannins (see section 6.1).

7.3 **Conclusions.**

A [C14]-labelled chitin precursor ([C14]-NAGA) and a tritiated amino acid ([H3]-cystine dihydrochloride) were incorporated preferentially in the anterior section of the PM immediately after feeding. Measurement of incorporation at subsequent times after a single feed indicated a pulse of peak radioactivity passing posteriorly in the PM (Chapter 4). The rate of production of the PM in the first four hours after feeding could thereby be calculated to be 5-7 mm/hr (Chapter 4). In male adult cockroaches under the conditions of the radiolabelling experiment, food entered and filled the PM only after a newly secreted PM had lined most of the midgut, but when the cockroaches had not been injected with radiolabel food entered the midgut just after secretion of PM had recommenced (Chapters 4 and 3 respectively). This seems to indicate that the injection of radiolabel caused a burst of production of PM, and a similar phenomenon resulted from injection of dyes (Chapter 3). It would appear therefore, that production is stimulated both by feeding and by humoral stimuli, e.g. perhaps

change in haemolymph osmolarity.

As decribed previously (Mercer and Day, 1952), the PM is a chitinous lamellate sheath with hexagonally orientated grid layers although orthogonally orientated grid systems were also be observed in this study, e.g. in the ectoperitrophic surface layer both types of grid system could be observed together (Chapter 5). The PM is closely associated with the oesophageal invagination and appears capable of considerable plastic deformation in this region (Chapters 2 and 5).

Where the PM is adpressed to the oesophageal invagination, the fibrils of the ectoperitrophic surface layer appear poorly organized. Immediately posterior to the oesophageal invagination, a grid system is clearly visible as the ectoperitrophic surface. About 2-3 mm posterior to this region, the ectoperitrophic surface appears to be overlaid with matted microfibrils deposited on the grid layer. Except in unusual circumstances (as indicated below), the matted microfibrils are not readily seen, however, since a granular deposit normally fills its interstices giving it a largely amorphous appearance (Chapter 5). The granular layer could be, in part at least, aminopeptidases immobilised on the ectoperitrophic surface layer (Peters and Kalnins, 1985).

In Chapter 2, PMs were described from some insects that ingested tannic acid, in which the main bulk of PM layers

appeared to change structure from a "fluid" PM to a more substantial and brittle PM, apparently moulded on the bulbous tip of the oesophageal invagination (Figure 2.2) giving a sinuate appearance. Overlying this sinuform PM was also a fine, smooth layer which, although not investigated with the electron microscope, was presumed to correspond to the amorphous ectoperitrophic surface layer and any closely associated fibrillar systems. It should be noted, however, that in these specimems the fine, smooth layer could not be observed to continue anterior to the oesophageal invagination, nor was the matted fibrillar ectoperitrophic layer; both this and the apparent different composition of these layers from the rest of the PM suggest that cells at or near the base of the caeca, secrete these outermost layers of the PM. This would also explain Lee's observation of PM producing cells in that region of the midgut (Lee, 1968). But even with some separation of origin of the ectoperitrophic layers from the other layers that make up the bulk of the PM, secretion of the PM as a whole would seem to conform to a Type II production by secretion from cells of the anterior region of the midgut.

In most of the insects that ingested polyphenols, the PM at first looked normal, but SEM investigation showed that ingestion of tannic acid, and to a lesser extent, gallic acid, had an effect on the amorphous layer, i.e. prevention of the deposition of the granular substance leaving the matted microfibrils visible (Chapter 5). When PM sections were

immersed in solutions of the above polyphenols, the *in vitro* effects of these polyphenols (i.e. surface deposits) were most marked in acidic solutions (Chapter 5). The necessity for acidic conditions to produce *in vitro* surface deposits could explain the apparent dissimilarity of *in vitro* and *in vivo* effects of gallic and tannic acid, since the midgut of the cockroach is presumed to be neutrally buffered (Bignell, 1982). The *in vivo* affect of tannic acid appeared not to affect permeability of the PM (Chapter 6) although it may affect protein digestion (see above and section 6.4). Tannic acid in the diet did appear to decrease consumption, ECD and faecal weight (Chapter 3).

NaCl solutions had a marked effect on the permeability of PM sections *in vitro*, increasing the hydraulic conductivity by 150% when the PM section was bathed in saline as opposed to water. The hydraulic conductivity and flux of protein-dye complex (molecular weight approximately 70,000 Dalton) through PM sections bathed in water were increased about 10-fold when the bathing solution was changed to saline. These effects were reversible. Hydraulic conductivity also increased when smaller, charged dyes perfused the PM section suggesting that ionic composition *in vitro* influenced permeability (Chapter 6). This could perhaps also apply *in vivo* and may provide the insect with a potentially powerful means of regulating movement of solutes, especially high molecular weight species, through the PM.

The PM appears to act mainly as a microfilter in *P. americana* to separate particles from solution. This in itself may have diverse effects as discussed above, but at the very least, may enhance the absorptive capacity of the midgut by creating a clear space next to the microvilli, thus allowing a greater solute to surface area ratio than might otherwise be obtained.

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