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MICROPUNCTURE STUDIES OF FORMED-BODY SECRETION BY THE EXCRETORY ORGANS OF THE CRAYFISH, FROG AND STICK INSECT

By J. A. RIEGEL
Zoology Department, Westfield College, University of London

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INTRODUCTION

In the course of investigations of the formation of urine by the antennal gland of the crayfish it was discovered that this process involved the secretion of formed bodies. The appearance and partial chemical analyses of the formed bodies of the crayfish are reported on elsewhere (Riegel, 1966). In the present report the lipid character of the walls of the formed bodies will be established, as will the fact that they contain digestive enzymes. Further, the results of similar studies on other excretory organs will be presented.

A number of lines of evidence prompted the writer to suspect that formed bodies are to be found in two organs whose structure and function have been well studied. These are the vertebrate glomerular nephron and the insect Malpighian tubule. The proximal portions (coelomosac and labyrinth) of the antennal gland show striking histological similarities to the proximal portions (renal capsule and proximal tubule) of the vertebrate nephron. This is especially true when electron micrographs are compared (Anderson & Beams, 1956; Farquhar & Palade, 1960; Kümmel, 1964; Pease, 1955). Furthermore, both organs have much in common functionally. For example, the dyes Congo red and phenol red are accumulated by cells of the proximal portions of both, as is albumen. Further, the potassium concentration is elevated above blood levels (Oken & Solomon, 1960; Riegel, 1965). Unpublished work indicates that bound potassium is associated with the formed bodies in the crayfish urine. This may be an explanation of the elevated potassium levels in the proximal portions of both types of excretory organ.

Electron micrographs of the crayfish antennal gland and insect Malpighian tubule also show striking similarities (Beams, Tahmisian & Devine, 1955; Wigglesworth & Salpeter, 1962). Furthermore, as established by Ramsay (1955*b*), the potassium concentration within the Malpighian tubule of *Dixippus morosus* is high compared with haemolymph levels.

MATERIALS AND METHODS

To verify the presence or absence of formed bodies in the vertebrate nephron and insect Malpighian tubule, those organs were sampled as follows. The kidneys of specimens of *Rana temporaria* were removed and placed in frog Ringer. The neck segment and proximal tubule were located and micropunctured using methods essentially similar to those used on the crayfish, except that the procedure was

completed in Ringer. Similarly, the Malpighian tubules of specimens of *Dixippus morosus* were dissected in stick-insect Ringer (Ramsay, 1955*a*). They were then transferred to liquid paraffin. The proximal (opaque) portion of the tubule was punctured. The micropuncture samples from the frog and stick insect were viewed under the microscope as described elsewhere (Riegel, 1966).

The foregoing methods were used for the routine collection of urine. However, it was necessary to establish that the occurrence of the formed bodies was not caused by some aspect of the isolation procedure (see Discussion). To check that the formed bodies were not artifacts, the various excretory organs were punctured, *in situ*, under conditions that were as 'natural' as possible.

Crayfishes were immobilized by wrapping them in a moistened 'Kimwipe' and placing them on their sides on the stage of a dissecting microscope. The fronto-lateral portion of the exoskeleton was carefully chipped away with a fine scalpel. This procedure exposed the intact hypodermis and the underlying antennal gland. If done carefully there was virtually no blood loss in this procedure. The labyrinth and proximal tubule of the antennal gland were punctured by pushing the pipette through the hypodermis.

Stick insects were immobilized in a Petri dish containing a 'Kimwipe' moistened with stick-insect Ringer. They were prevented from moving the head and thorax by placing strips of plasticene over those parts of the body. The Malpighian tubules were exposed by a mid-dorsal incision along the hinder one-third of the abdomen. The flaps of the incision were held apart and the abdomen was held steady by a pair of forceps whose tips rested on the flaps on either side of the hindgut and rectum. As far as could be ascertained this procedure resulted in no damage either to the Malpighian tubules or to their tracheal supply. The tubules (opaque and translucent portions) were punctured directly after dissection. In some cases the preparation was immersed in liquid paraffin after dissection. In other cases the dissection was made under liquid paraffin.

Frogs were pithed and placed on a moistened 'Kimwipe' on the stage of a dissecting microscope. The viscera were exposed by removing the ventral abdominal wall, care being taken to leave the ventral abdominal vein intact, and the viscera overlying the kidneys were pushed to one side. When female frogs were used, it was necessary to sever the mesovarium, which was done with no blood loss. In general, micropuncture samples were taken from the mesial portion of the anterior one-third of the kidneys. This portion seemed to be relatively free of large blood vessels which impeded the view. In most cases it was necessary to sever the peritoneal sheath over the kidneys, since the elasticity of that structure tended to prevent accurate placement of the pipette. Samples were taken only during the period in which blood flow remained 'normal'. This could be seen as a rapid movement of blood in the large renal veins.

Samples removed from the various excretory organs by the above procedures were viewed under the microscope as described earlier.

The histochemical method of Gomori (cited in Culling, 1963) was used to identify lipid in the formed bodies. Micropuncture samples of *c.* 0·01–0·1 μl. were placed on microscope slides. The larger samples (> 0·05 μl.) were smeared in the way used to make blood smears. A straight-edged fragment of a coverslip was used as a smearing

tool. The slides were air-dried rapidly and placed in a saturated solution of Sudan III or Sudan Black B in 70% ethyl alcohol. After staining and differentiating, the smear was mounted in glycerine and covered with a coverslip.

Attempts were made to detect enzymes within the formed bodies by histochemical means. Tests were made for acid phosphatase using the methods of Gomori and Rutenberg & Seligman (cited in Culling, 1963). These were totally negative. Tests for alkaline phosphatase, using the azo-coupling method outlined by Culling (1963), were partially successful. However, it was decided to make use of more direct method to test the enzymatic activity of the formed bodies.

The method of Gates (1927) was used to test for the presence or absence of proteolytic activity in the formed bodies. Gates' method consists essentially of noting the digestion (clearing) of exposed and developed photographic film on which the tissue extract is placed. For the purposes of the present experiments the photographic film was cut into pieces having gelatin surfaces of *c.* 0·01–0·03 mm^2. These pieces were placed under liquid paraffin in siliconed watch glasses. Then micropuncture samples (*c.* 0·01–0·2 μl.) were deposited on the surfaces of the pieces. The reduced silver grains in the gelatin made it easy to follow the progress of digestion. The liquid of the sample became progressively darker and when digestion was complete all that remained of the film was the small piece of celluloid backing.

Tests were made of the proteolytic activity of samples of urine removed from the coelomosac, labyrinth and distal tubule of the crayfish. The proteolytic activities of urine from the Malpighian tubule and urine from the proximal tubule of the frog were also tested. The procedure was as follows. Four gelatin (photographic film) pieces of approximately equal size were placed in a watch glass under liquid paraffin. Samples of urine which had been collected previously were placed on three of the pieces. A sample of acid NaCl (200 mM/l. NaCl made to pH 6 with HCl) was added to the fourth piece and to the first piece of film. After 15 min. distilled water (neutralized with $NaHCO_3$) was added to the first and second pieces. All reactions were run at room temperature (20–21° C.). The procedures may be summarized as follows:

Number	Procedure
1	Gelatin + sample + acid NaCl + neutral distilled water
2	Gelatin + sample + neutral distilled water
3	Gelatin + sample
4	Gelatin + acid NaCl (control)

RESULTS

As shown in Pl. 1, B–C, formed bodies are present in urine removed from the frog nephron and from the insect Malpighian tubule. The formed bodies of the frog and insect appear to be similar to the spheroids found in the crayfish (Pl. 1, A). Urine samples removed from the various excretory organs under carefully controlled conditions contained formed bodies. These appeared to be similar to the formed bodies found in urine removed by the routine procedures. The three procedures used for obtaining samples from Malpighian tubules *in situ* appeared to make no difference to the appearance of the formed bodies.

The formed bodies removed from all three excretory organs stained intensely

with Sudan Black B after only 5 min. exposure. They also stained with Sudan III, but this required periods up to an hour.

The proteolytic activities of the formed bodies from the various excretory organs are summarized in Table 1. As shown in the table, the most immediate reaction is seen when the formed bodies are disrupted by distilled water after prior treatment with acid NaCl (column 1). In the case of the labyrinth samples acid media had little effect, but alkaline distilled water (pH = 7·6) had an accelerating effect on the digestive reaction. Alkaline distilled water immediately halted the digestive action of the formed bodies from the Malpighian tubule. The reactions were weakest in the samples containing formed bodies but otherwise untreated (column 3). This suggests that the digestive enzymes are almost entirely associated with formed bodies and not with the medium which surrounds them.

Table 1

Summary of the proteolytic reactions of formed bodies in urine removed from the coelomosac (COEL), labyrinth (LAB) and distal portion of the distal tubule (DDT) of the crayfish antennal gland. The abbreviation, MPT, refers to urine samples removed from the Malpighian tubule of the stick insect. The abbreviation, PROX, refers to urine samples removed from the proximal tubule of the frog nephron. The column numbers refer to the procedures listed in the methods section. Reaction symbols are as follows: −, no reaction after 4 hr.; +, reaction detected after 2 hr.; ++, reaction detected after one hour; +++, immediately detectable reaction which went to completion within one hour. DW = distilled water.

	1	2	3	4	Remarks
COEL	++ to +++	−	−	−	−
LAB	− to +	− to +	+	−	Alkaline DW, +++
DDT	+ to ++	+ to +++	+	−	−
MPT	+++	++	+	−	Alkaline DW, −*
PROX	+++	−	+	−	−

* The reaction was halted immediately on the addition of alkaline distilled water, but not when neutral distilled water was added.

DISCUSSION

The presence or absence of vesicles in the excretory organs of various animals was the subject of considerable controversy in the latter half of the nineteenth century. By the early twentieth century, however, there was general agreement that the evidence for vesicular secretion was based largely on artifacts (see Wigglesworth, 1931; Marshall, 1934; and Smith, 1951 for reviews). In the present study urine samples have been removed from the excretory organs of animals under conditions which were as 'natural' as it seems possible to make them. It is therefore unlikely that the formed bodies observed here are artifacts.

It is a source of puzzlement why the formed bodies here described have never been closely investigated. It is possible that earlier arguments concerning the artifactual basis have acted to deter such enquiry. On the other hand, it is possible that the formed bodies have not been observed previously in fresh material. In the case of the Malpighian tubule the formed bodies are easily obscured. Urine removed from the opaque portion of that structure is packed with matter, much of which appears to be crystalline. However, when the sample is placed on a siliconed microscope slide under liquid paraffin it remains as a rounded droplet, and under these

conditions, the crystalline material sinks to the bottom of the droplet and remains motionless; the formed bodies remain suspended in the liquid of the drop and they are affected by Brownian motion.

It does not seem to be speculating too wildly to suggest that formed bodies will be found to underly a very large number of secretory processes. The production of formed bodies would seem to be a natural corollary to pinocytosis, which, of course, is to be found in a wide variety of cells (Chapman-Andresen, 1962).

Furthermore, the results of this study bear heavily on many aspects of excretion in animals. In fact, some of the current concepts of that process may be in need of revision. For example, it is well known that the cells of the proximal tubule of the glomerular nephron accumulate substances, such as egg albumen and horse-radish peroxidase (Novikoff, 1961). The view is held currently that such substances are withdrawn from the presumptive urine after they have undergone a process of ultrafiltration in the renal capsule. However, on the basis of the present results it seems more likely that such substances are accumulated by the proximal tubule cells from the blood. Probably they or their digestion products are then released into the urine within formed bodies.

It is possible that a wide variety of substances can be secreted without the necessity of invoking special secretory mechanisms or 'carriers' for them. Certainly, it is difficult to see how cells of the vertebrate proximal tubule could have evolved special mechanisms to handle horse-radish peroxidase or egg albumen. It is possible that even the renal test substance, inulin, is secreted. Falbriard, Schaller & Simon (1963) have shown that inulin can be accumulated in vacuoles within the cells of the glomerulus and proximal tubule. Finally, the results of the present study open the possibility that the only materials which are actually filtered are molecules of low molecular weight. Certainly, the ability of epithelial cells to accumulate large molecules and release them into the urine would greatly decrease the necessity for invoking grossly porous properties to membranes proximal to the cells of the visceral layer of the renal capsule (see reviews by Ullrich & Holmes, 1963; and Chinard, 1964).

The results of the present study should provide a basis for a better understanding of the mechanism underlying urine formation by the vertebrate aglomerular kidney. According to Marshall (1934), the nephrons here consist of tubules which correspond only to the proximal tubules of the glomerular kidney. It is suggested that urine formation in the aglomerular kidney is initiated by the secretion of formed bodies.

According to the scheme of Ramsay (1958 and earlier), urine formation by the insect Malpighian tubule proceeds by the secretion of potassium into the lumen of the tubule. However, it is possible that what Ramsay believed to be potassium secretion is actually the secretion of formed bodies. As mentioned earlier, the formed bodies of the crayfish antennal gland have significant quantities of bound potassium associated with them. Formed-body secretion might also explain why the isolated Malpighian tubules would not function properly without serum from the stick insect as a component of the medium. Assuming that the mechanism for secretion of the formed bodies depends upon pinocytosis, it would be expected that an inducer would be necessary. It is possible that only the serum of the stick insect would contain the proper inducer.

The present results do nothing to weaken Ramsay's main hypothesis, that the

primary urine of the insect is formed by secretion. In fact, that hypothesis is strengthened. However, Ramsay's conclusion that organic molecules (amino acids, sugars) are not secreted but enter the lumen of the tubule by passive diffusion undoubtedly requires further investigation.

In the work of Wigglesworth & Salpeter (1962) it was seen that small spheres (0·1 μ and larger) were discharged from the cells of the upper portion of the Malpighian tubule. Those investigators thought that the spheres served as crystallization nuclei for uric acid in the distal portion of the tubule. In view of the present results, it is more likely that the spheres seen by Wigglesworth & Salpeter are the spheroids described herein.

SUMMARY

1. Formed bodies, similar to the spheroids of the crayfish antennal gland, are present in urine removed from the frog nephron and from the insect Malpighian tubule by micropuncture.

2. The formed bodies contain digestive enzymes and appear to function in the digestion of materials accumulated by the cells of the proximal portions of these excretory organs.

3. These results indicate that some concepts regarding the process of urine formation in animals are in need of reappraisal. It is possible that in the crayfish and other animals with filtration kidneys only very small molecules are filtered. In the Malpighian tubule the secretory basis of urine formation may be more firmly established. However, whether or not organic materials enter the urine by passive diffusion may be in need of reinvestigation.

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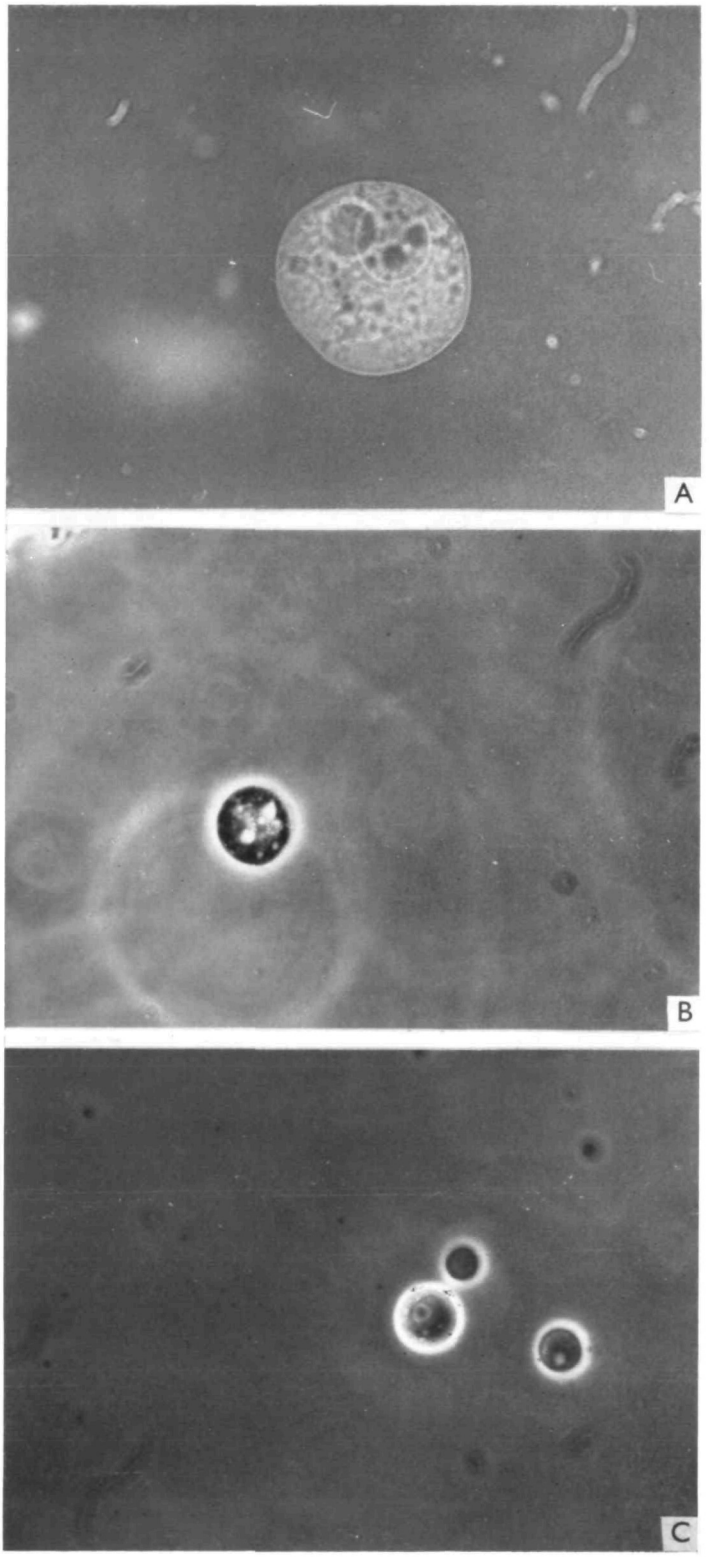
A
B
C

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EXPLANATION OF PLATE

Formed bodies removed from the excretory organs of the crayfish (A), frog (B) and stick insect (C). All were stained with Sudan III. Photomicrographs were taken under phase-contrast illumination at 500×. The formed bodies swelled appreciably in the glycerine mounting medium.