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THE LABIAL GLAND: A SALT-SECRETING ORGAN OF SATURNIID MOTHS

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The labial glands of saturniid moths are two exceedingly convoluted tubules, 100–150 mm. long and only 150–200 μ in diameter, located in the ventral part of the thorax (Edwards, 1964; Kafatos & Williams, 1964). The proximal ends of the two glands unite and open to the surface through a pore in the middle of the labium. Through this opening the insect releases a voluminous liquid at a precise time during ecdysis of the adult moth. In *Antheraea pernyi* and *A. polyphemus* the fluid not only serves as a solvent for the cocoon-opening enzyme, cocoonase, but also maintains an optimal pH for this enzyme's attack on the cocoon (Kafatos & Williams, 1964; Kafatos, Tartakoff & Law, 1967). Since the total amount of fluid greatly exceeds the volume of the glands, it must be swiftly elaborated from the haemolymph.

The morphology of the labial glands (Kafatos, 1968; Kafatos & Feder, 1968) suggests that elaboration of the fluid is an active process. The wall of each tubule consists of approximately 1000 giant polyploid cells, all of the same type, arranged in a single cylindrical layer. The cells show a striking resemblance with those encountered in the renal tubules and salt glands of vertebrates and in other actively transporting tissues; they are characterized by extensive cellular plications, which greatly increase surface area and bring abundant mitochondria into intimate proximity to the plasma membrane (Sjöstrand & Rhodin, 1953; Copeland, 1964; Anderson & Harvey, 1966). The gland as a whole is deeply enmeshed in a complex of tracheae and tracheal air sacs, each cell being provided with an unusually dense network of tracheolar ramifications.

The present report provides physiological evidence that the labial gland is, in fact, engaged in an active process of salt secretion, possibly centred around the active accumulation of blood potassium through an exchange of potassium and hydrogen ions.

MATERIALS AND METHODS

A. *Animals*

Pupae of *Antheraea pernyi* were removed from storage at 3–6° C., taken out of their cocoons, and allowed to develop into adults at room temperature. The moths were used around the time of ecdysis: no earlier than complete resorption of the moulting fluid and no later than 5 hr. post-emergence. A few experiments were performed on the related saturniids *A. polyphemus* and *Hyalophora cecropia*.

B. *Collection of fluid*

As first observed by Dr Richard A. Lockshin, secretion could be provoked by the injection into the thorax of the moth of approximately 0·3 μmole physostigmine (e.g. 15 μl. of 0·01 M physostigmine sulphate in water). Since the blood volume is about 1·5 ml., the final concentration was approximately 5×10^{-4} M. Most rapid secretion was elicited by an amount of physostigmine which caused the moth to be barely incapable of locomotion. At this critical concentration the moth was unable to right itself when placed on its back, but showed vigorous and repeated contractions of the abdomen and either copulatory motions or egg-laying. Secretion was impaired by doses high enough to immobilize or small enough to permit semi-coordinated locomotion. Injected moths were placed on their back and the fluid was collected in glass capillary tubes (Drummond Scientific Co., 0·6 mm. I.D.).

In one group of moths secretion was studied as a response to confinement in the cocoon rather than to physostigmine injection. The cocoon had previously been cut in two and the animal removed temporarily. The anterior half cocoon was coated internally with a layer of melted wax, and a glass capillary was inserted into the apex and secured with more wax. Just before ecdysis the cocoon was reassembled with the moth inside and head-end down. The secretion simply rolled into the capillary, from which it was collected and studied.

C. *Blood determinations*

Samples of whole blood were collected in capillaries by pricking either the abdomen or the thorax. Blood volumes were estimated by the dilution of injected salts (NaCl, $Na^{36}Cl$); on the average the volume (in ml.) was equal to $50 \pm 10\%$ of the weight (in g.) of the freshly emerged moth, after discharge of the meconium. The proportion was somewhat higher for males (55%) than for females (45%).

D. *Analytical methods*

Cations were determined by a Model 303 Perkin–Elmer atomic absorption spectrophotometer. The method of Fiske & Subbarow (1925) was used for measurements of phosphate. Chloride was estimated by a micromethod adapted from the usual Mohr procedure (Kolthoff & Sandell, 1952); samples were mixed in a white Pyrex depression slide with a drop of indicator (0·1% K_2CrO_4, pH about 8·0) and titrated with 0·010 M-$AgNO_3$ delivered from a microburette, until the precipitated Ag_2CrO_4 failed to dissolve upon stirring. Satisfactory results were obtained only in the absence of proteins or other silver-insoluble anions. For brevity, all ionic concentrations have been expressed in millimolar units (mM) in this report, instead of the more correct g. ions/litre.

The bicarbonate content of secretion was assayed in two ways: by the syringe method of Scholander & Roughton (1943) for the microgasometric estimation of total CO_2, and by comparing titration curves of secretion and bicarbonate solutions. The titrations were performed with 1 ml. samples stirred by a slow stream of N_2. Acid was delivered from a microburette calibrated to 0·2 μl. and the pH was measured with a combination of glass electrode and calomel electrode attached to a pH meter with expanded scale (Model 135, Instrumentation Laboratory).

Measurements of pH on very small samples (*ca.* 10 μl.) were performed with a microelectrode assembly (Beckman no. 40317). When larger volumes were available, a one-drop electrode with a capacity of 0·2 ml (Beckman no. 40278) was used.

Osmotic pressure was estimated by comparison with NaCl standards, from the time required for melting of frozen samples placed in slowly warming brine (Gross, 1954). A few microlitres were sufficient for each determination.

E. *Electrical potentials*

Two glass electrodes, consisting of capillaries filled with concentrated KCl, were positioned as described under Results and connected to calomel electrodes through agar-KCl bridges. The potential difference was read from a potentiometer (Radiometer PHM4).

F. *Injections into haemolymph*

Injections were performed under CO_2 anaesthesia by means of a 27-gauge hypodermic needle inserted into either the thorax or the abdomen. Isotopes were obtained from New England Nuclear Corporation. ^{22}Na was counted in a gamma counter; ^{36}Cl and ^{14}C by liquid scintillation with aqueous scintillator fluid (Law, Zalkin & Kaneshiro, 1963).

RESULTS

A. *Rates of secretion*

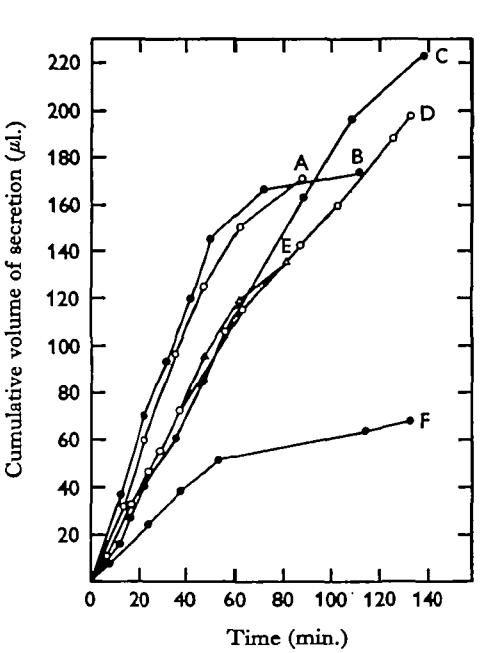
(1) *Secretion in response to physostigmine.* Secretion proved to be influenced by many factors. Both the rate and the total volume are limited as long as the pharate moth remains moist with moulting fluid. Secretion becomes maximal at about the time of ecdysis and declines thereafter. In a typical *A. pernyi* over 200 μl. can be collected if the moth is challenged with physostigmine injection at the moment of spontaneous ecdysis, but only 30–40 μl. if injection is postponed for 10 hr. After 15 hr. less than 5 μl. can be collected. Not only ageing, but also injury, poor health and dessiccation impair secretion (Fig. 1, F).

Under optimal conditions, pernyi moths of normal weight (*ca.* 3 g. (♂) and 5 g. (♀)) secrete 1·5–3·5 μl./min. (Fig. 1). This rate is maintained for about 1 hr. and then slowly declines. The total volume may reach 300 μl. In one case a male moth weighing 2·5 g. secreted 270 μl. of fluid within 2 hr., corresponding to about 10% of its total weight or 20% of its estimated blood volume. This is an extraordinary performance, considering the dimensions of the average labial gland: a length of 100–150 mm., a diameter of 150–200 μ and a wall thickness of 25–30 μ (Kafatos & Feder, 1968). Thus, the combined volume of all cells in the two tubules can be estimated as 2–5 μl.—scarcely greater than the volume of fluid which these cells secrete in one minute.

(2) *Secretion in response to confinement.* Secretion rates were determined for moths which were stimulated, not by physostigmine, but simply by confinement in their cocoon during ecdysis. Figure 2 records the rates observed in four such animals. The results were essentially indistinguishable from those obtained when secretion was provoked by physostigmine (Fig. 1).

(3) *Secretion in partially dissected animals.* Whether induced by physostigmine or by confinement, secretory activity coincides with a period of vigorous muscular contrac-

tions in the thorax and abdomen, resulting in high blood pressure (Lockshin & Williams 1965). Experiments with partially dissected moths revealed that this blood pressure may facilitate secretion, but is by no means prerequisite for it. Thus, following excision of the abdomen in *A. pernyi* or *A. polyphemus*, secretory activity continued, albeit at a decreased rate: 20–50% of normal immediately after amputation, and 5% an hour later. Similar incomplete inhibition resulted from the removal of the longitudinal flight muscles, the major muscles of the thorax. In fact, the glands continued to secrete at a rate 1–5% of normal even when partially isolated by removal of the surrounding muscles, as well as of the abdomen, wings and legs.



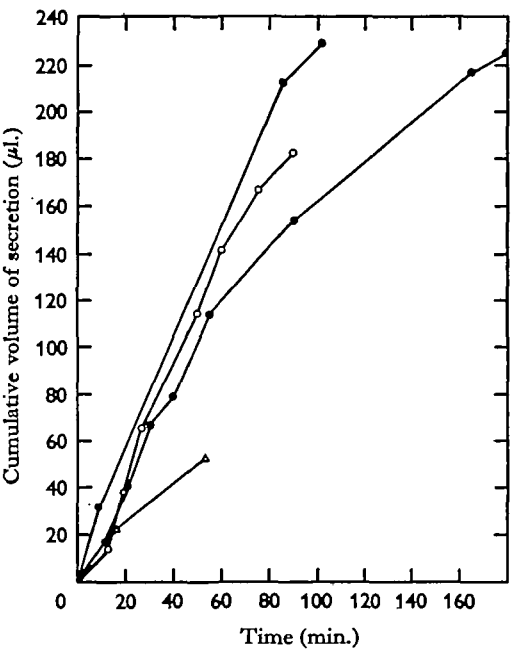


Fig. 1. (left). Rate of secretion under the influence of physostigmine. *A. pernyi* were injected with 3 μl. of 0·05 M physostigmine sulphate, just before spontaneous ecdysis. Time was counted from the first appearance of fluid in the facial region. In all cases secretion continued past the end of the measurements. Animal A: ♀, 5·5 g.; B: ♂, 2·9 g.; C: ♀, 4·7 g.; D: ♂, 3·0 g.; E: ♀, 5·5 g.; F: ♂, 1·6 g. (animal desiccated after prolonged storage in the cold).

Fig. 2. (right). Rate of secretion during mechanical confinement. Data obtained from four *A. pernyi*, confined in wax-lined cocoons just before ecdysis, without injection of physostigmine (cf. Fig. 1). Time was counted from the first appearance of fluid in the capillary inserted in the anterior end of the cocoon. For further details see text.

B. *Composition of normal secretion and comparison with blood*

From the dimensions given above it can be calculated that the lumina of both tubules have a combined capacity of only 3–5 μl.—barely sufficient for storing the amount of fluid released within 2 min. Clearly, the gland must elaborate the bulk of the secretion from the surrounding haemolymph. Thus, a detailed comparison between secretion and blood is desirable.

(1) *Cations.* Table 1 records typical analyses for the major inorganic cations, K^+, Na^+, Ca^{2+}, and Mg^{2+}, in haemolymph and in the total secretion from the same

moths. Obviously, the gland accumulates K^+ and tends to exclude, in order of increasing efficiency, Na^+, Ca^+, and Mg^{2+}.

The contrast between K^+ and the other cations is even more striking when one considers only 'late secretion', collected some time after the first release of fluid (Table 2). The very first drops of secretion, which presumably include fluid stored in the lumen before stimulation, are always more similar to blood than is late secretion. Clearly, it is late secretion that reflects more accurately the activity of the stimulated gland.

Table 1. *Comparison of cations in blood and in total secretion*

Moth sex and weight (g.)	*	K^+	Na^+	Ca^{2+}	Mg^{2+}	Volume of secretion collected (μl.)	Time for secretion (min.)	Secretion rate (μl./min.)
♂, 2·9	S	184	0·53	0·046	0·17	165	76	2·2
	B	38	4·0	13	75			
	S/B	4·9/1	1/7·6	1/280	1/230			
♂, 1·6	S	225	0·35	0·55	0·30	52	57	0·9
	B	43	3·8	7·5	45			
	S/B	5·2/1	1/11	1/14	1/150			
♀, 4·6	S	169	0·28	0·06	0·17	162	91	1·8
	B	39	1·5	6·0	38			
	S/B	4·3/1	1/5·4	1/100	1/220			
♂, 3·0	S	182	0·47	0·06	0·15	141	80	1·8
	B	31	2·8	6·5	43			
	S/B	5·9/1	1/6·0	1/110	1/290			
Av.	S	190	0·41	0·18	0·20	130	76	1·7
	B	38	3·0	8·2	50			
	S/B	5/1	1/7·3	1/46	1/250			

* S: overall concentration (mM) in secretion collected during the indicated time. B: concentration (mM) in a sample of haemolymph collected after the secretion. S/B: ratio of concentrations in secretion and blood.

Note: In calculating S/B, B has been taken as unity in the case of K^+, and S for all other ions. Thus, the numerator and denominator indicate, respectively, the level of ion accumulation or exclusion by the labial gland.

Table 2. *Comparison of blood, early secretion and late secretion**

Ion	Blood (mM)	Early secretion mM	Early secretion S/B	Late secretion mM	Late secretion S/B	Stored Fluid† S/B
K^+	45	248	5·5/1	244	5·4/1	5·9/1
Na^+	3·7	0·70	1/5·3	0·074	1/50	1/1·2
Ca^{2+}	6·0	1·1	1/5·5	0·017	1/350	1/1·1
Mg^{2+}	39	0·18	1/220	0·014	1/2800	1/46

* Data from a single *A. pernyi*, ♂, 3 g. Early secretion: first 20 μl. Late secretion: 60 μl. collected after the first 50 μl. Blood: haemolymph sample collected after 'late secretion'.

† Calculated by assuming that early secretion consists of 16 μl., of late secretion plus a volume of stored fluid equal to the capacity of the gland, 4 μl.

The accumulation ratio [secretion]/[blood] (S/B; concentration in secretion divided by concentration in blood) can be used as a convenient index of gland function. S is proportional to the net ion flux into the lumen; thus S/B indicates the selectivity

of the gland, i.e. how fast the various ions would be secreted if they all were equally concentrated in blood. According to the data in Table 2, K^+ can be accumulated at a rate as much as 270 times faster than Na^+, 1900 times faster than Ca^{2+}, and 15,000 times faster than Mg^{2+}.

(2) *Anions*. Assays with a sensitivity of 0·03 mM failed to detect phosphate in the secretion of *A. pernyi* and *H. cecropia*. By contrast, the blood phosphate was more than 100 times higher: in *A. polyphemus* it is reported as 4–22 mM (Carrington & Tenney, 1959), and in *A. pernyi* it was estimated as 4 mM.

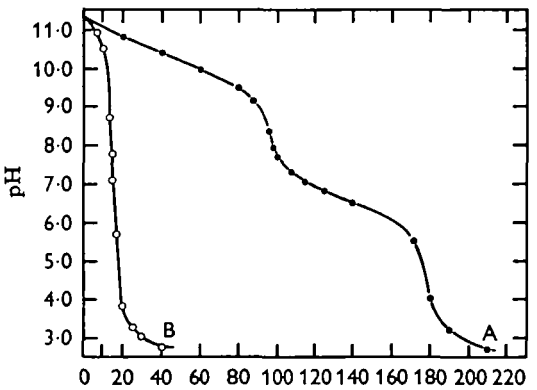


Fig. 3. HCl-titration curves of labial gland secretion. Curve A (—●—): late secretion, diluted with 17 parts of distilled water, and brought to pH 11·3 with KOH; the carbonate content of the diluted sample was estimated as 0·010 M, by comparison with standard $KHCO_3$ solutions. Curve B (—○—): the same sample, after acidification, brief boiling to remove CO_2, and adjustment of pH to 11·3 with KOH.

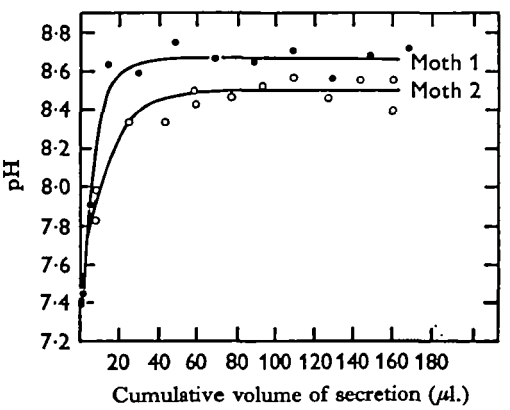


Fig. 4. pH of secretion, measured in sequential drops collected from two different male pernyi moths.

Both the early and late secretion of male pernyi moths contained 19·3 ± 1·4 mM-Cl^-. In female moths the corresponding value was 10·3 ± 2·4 mM. Because of protein interference no direct estimate of blood Cl^- was possible. However, the just-mentioned values are in line with the 21 mM reported by Carrington & Tenney (1959) for the haemolymph of *A. polyphemus* (pooled sample from both sexes).

In gasometric analyses total CO_2 in the secretion was estimated as 170 ± 20 mM—much higher than in blood. At a pH of 8·5 essentially all this CO_2 must be in the form of HCO_3^-. A similar value (180 mM) was calculated by titration. Titration curves of authentic secretion (Fig. 3, A) were identical with the curves of equimolar bicarbonate solutions. Upon acidification and heating the secretion lost essentially all its buffering capacity (Fig. 3, B), confirming that no appreciable amount of buffering ions other than HCO_3^- is secreted by the gland.

In sum, HCO_3^- and Cl^- together amount to 180–200 mM, thereby paralleling the cation concentration (190 mM-K^+) within experimental error.

(3) *pH.* Pooled samples of secretion had a pH of 8·4–8·7 (more rarely 8·3 or 8·8). Pure bicarbonate solutions have a pH of 8·3 when freshly prepared, but rapidly become more alkaline, because of CO_2 evolution. Such alkalinization undoubtedly occurs during collecting and storage of the secretion, which corresponds to a 180 mM solution of $KHCO_3$. The pH of the secretion (8·5), compared to that of adult blood (6·5; Carrington & Tenney, 1959), implies that the gland maintains a hundredfold gradient of H^+ and OH^-.

Sequential drops of secretion from individual animals were assayed with the micro glass electrode (Fig. 4). This experiment demonstrated that the pH is essentially constant throughout secretion. Only very early samples of fluid deviate, being invariably less alkaline than the rest. This probably represents yet another similarity between blood and the stored liquid present in the gland before stimulation (§ B 1).

(4) *Osmotic pressure.* Haemolymph and secretion are both osmotically equivalent to about 0·18 M-NaCl. In direct comparisons between blood and secretion no significant difference could ever be detected within the limits of the method (*ca.* 10 m-osmoles).

C. *Composition of secretion after alteration of haemolymph composition*

(1) *Injections of unusual cations.* Li^+, Rb^+ or Sr^{2+} were made available to the labial gland by injecting the corresponding chloride salt (0·2 M, 150 µl.) into the haemolymph 1–4 days before ecdysis. Table 3 records typical results. Like K^+, Rb^+ was

Table 3. *Secretion of unusual cations*

			Early secretion		Late secretion	
Animal	Ion	Blood (mM)	mM	S/B	mM	S/B
1	K^+	41	150	3·7/1	160	3·9/1
	Rb^+	4·7	14	3·0/1	16	3·4/1
2	K^+	36	180	5·0/1	210	5·8/1
	Rb^+	2·2	10	4·5/1	8·4	3·8/1
3	K^+	75	350	4·7/1		
	Li^+	10·5	1·1	1/9·5		
4	K^+	51	190	3·7/1	190	3·7/1
	Sr^{2+}	2·4	0·15	1/16	0·026	1/92

secreted at a concentration higher than in blood; as indicated by S/B, it was accumulated with only slightly (but consistently) lower efficiency than K^+. By contrast, Li^+ and Sr^{2+} were relatively excluded from the secretion: Li^+ as if it were Na^+, and Sr^{2+} with an efficiency intermediate between that of Na^+ and Ca^{2+} (cf. Table 2). Here again, early drops of secretion were not as different from blood as were later samples.

(2) *Injections of* ^{22}Na. Small amounts of carrier-free $^{22}NaCl$ were injected into developing *A. pernyi*. Results were similar, irrespective of whether the isotope was administered as early as the first day of development or as late as the final day before ecdysis. The specific activity of late secretion (c.p.m./μl.) was 1/40 to 1/90 that of blood. This agreement with the usual S/B ratio for total Na^+ (1/50, Table 2) suggests that, after physostigmine stimulation, Na^+ enters the lumen from a compartment in exchange equilibrium with blood—probably from blood itself. Surprisingly, early samples of secretion had less radioactivity (only 1/50 of blood) than expected from their relatively high Na^+ content (S/B = 1/5, Table 2).

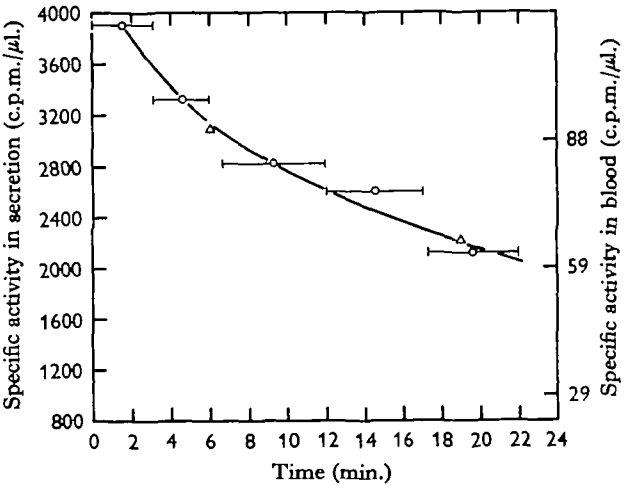


Fig. 5. Radioactivity in secretion and blood, following injection of $NaH^{14}CO_3$. Labelled bicarbonate was injected at time = 0 into the fourth abdominal segment of *A. polyphemus* already secreting under the influence of physostigmine. ⊢, secretion collected during the indicated time interval; O, mid-point of the interval; Δ, blood. Blood radioactivity was plotted on the expanded ordinate at right; the necessary expansion factor indicated that S/B = 34/1.

(3) *Injections of* $^{36}Cl^-$. Sixteen *A. polyphemus* were injected, each with 50 μl. of distilled water containing 0·4 μmole $Na^{36}Cl$ (0·5 μc./μmole). The specific activity (c.p.m./μl.) of blood and secretion was monitored for at least 1 hr. following physostigmine injection. The calculated value of S/B, 0·7 ± 0·3, indicated that chloride is nearly as abundant in the secretion as in blood. No difference was detected between males and females.

In an attempt to evaluate the effect of chloride concentration on flux of ^{36}Cl, S/B was determined before and after injection of 100 μl. of 0·5 M-KCl + 0·75 M-$MgCl_2$ into each of six *A. polyphemus*. This injection raised blood chloride eightfold on the average. S/B showed only a slight increase, from 0·7 ± 0·3 to 1·1 ± 0·3.

(4) *Injections of* $H^{14}CO_3^-$. Three physostigmine-stimulated *A. polyphemus* were injected, each with 50 μl. of distilled water containing 0·29 μmole of $NaH^{14}CO_3$ (5·9 μc./μmole). Most dependable results were obtained when the bicarbonate was rapidly mixed with circulating blood by injection into the abdomen. Even so, bicarbonate concentrations never attained a steady state, because of rapid loss via the

respiratory system; about one-half of the radioactivity was lost from the blood in less than 30 min.

Specific activity (c.p.m./μl.) in the secretion reached a peak between 1 and 3 min. after injection and declined thereafter. To calculate S/B, S and B were followed over a period of more than 20 min. (Fig. 5). In order for B data to fall on the same curve as S they had to be plotted on a scale expanded by a factor of 34. Therefore, S/B = 34/1; this value refers to *total carbonate*, since the injected bicarbonate could be expected to equilibrate rapidly with dissolved carbon dioxide in blood, much of it undissociated because of the low pH.

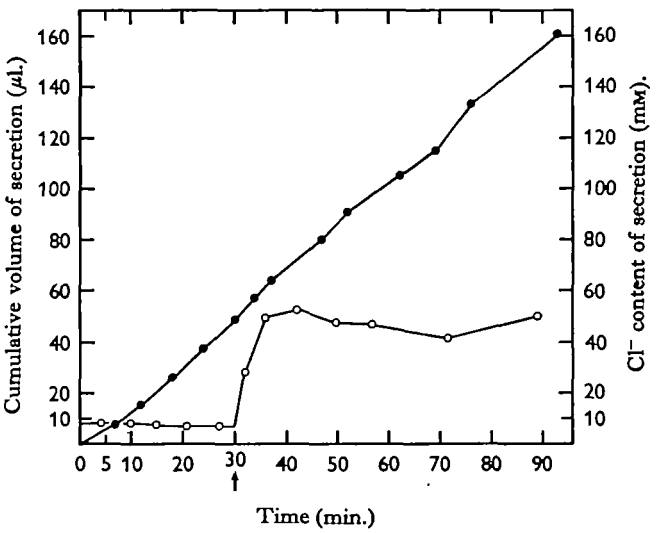


Fig. 6. Effect of chloride injection. An *A. pernyi* (♀, 3·8 g.) was injected with physostigmine; rate of secretion (—●—) and concentration of Cl^- in the fluid (—○—) were followed. Thirty minutes later (arrow), 40 μl. of 0·5 M-KCl + 0·75 M-$MgCl_2$ were injected into the haemocoel. The subsequent rise in the Cl^- content of secretion (*ca.* 40 mM) was equal to the calculated increase in blood chloride, assuming 1·9 ml. as the blood volume. The rate of secretion was unaffected.

(5) *Injections of* NaCl. By injecting concentrated NaCl solutions, it was possible to raise the Na^+ content of blood to as much as 100 mM. Invariably the S/B ratio for late secretion remained normal (approximately 1/50 to 1/100). In other words, over a nearly 50-fold range, the net Na^+ flux was roughly proportional to the Na^+ content of haemolymph.

The injected NaCl augmented the osmotic pressure of blood. As a result it caused a corresponding decrease in the volume of secretion and an increase in its K^+ concentration, presumably by opposing the osmotic flow of water (§C7).

(6) *Injections of* Cl^-. The chloride content of blood was enhanced by injecting a mixture of 0·5 M-KCl and 0·75 M-$MgCl_2$. The Cl^- content of the secretion rose rapidly to a new level (within 6 min.; Fig. 6), up to 280 mM in some experiments. Perhaps because of the added K^+ these injections failed to decrease the rate of secretion, despite the large increase in osmotic pressure of the blood.

Blood Cl^- was estimated indirectly, by assuming that the blood volume (in ml.)

was equal to 50 % of the moth's weight (in g). In all cases this estimate matched the concentration measured in the secretion (Table 4), suggesting that S/B for Cl^- remains roughly equal to 1 over a 12-fold range in blood chloride.

(7) *Changes in blood osmotic pressure.* Osmotic pressure was modified by injections of a variety of hypertonic or hypotonic solutions (Na_2HPO_4–NaH_2PO_4, NaCl, KCl, $MgCl_2$, sucrose). In each case an increase or decrease in blood osmotic pressure provoked a parallel change in the osmotic pressure and K^+ content of the secretion.

Table 4. *Chloride injections into blood*

Sex	Weight of moth (g).	Volume of solution injected (μl.)*	Estimated concentration of Cl^- in blood† (mM)	Cl^- concentration in secretion (mM)
♂	2·8	35	68	59
♂	1·6	100	240	280
♀	3·8	40	48	47

* 0·5 M-KCl and 0·75 M-$MgCl_2$, or 2000 mM-Cl^-.

† Estimated by assuming that the blood volume (in ml.) is equal to one-half the moth's weight (in g.), and ignoring any chloride loss to the tissues.

In a typical experiment samples of secretion and blood were obtained from a moth (♀, 4·5 g.). Then 20 μl. of a concentrated sodium phosphate buffer were injected (pH 6·5, about 6 osmoles), and new samples were collected. Before injection the osmotic pressures of blood and secretion were identical (*ca.* 0·32 ± 0·01 osmoles); after injection, they were higher (*ca.* 0·39 ± 0·01 osmoles), and again indistinguishable from each other. In fair agreement, K^+ in the secretion increased from 165 ± 10 mM to about 210 ± 15 mM.

In two other experiments sufficient saturated NaCl was injected to raise blood Na^+ to 94 and 84 mM, respectively, so as to establish blood osmotic pressures 0·18 and 0·16 osmoles above normal. The corresponding levels of K^+ in the secretion were approximately 100 and 80 mM above average, implying an increase of 0·20 and 0·16 osmoles, respectively, in the secretion.

In a fourth experiment a moth was bled and then injected with an amount of 0·05 M-KCl expected to decrease the osmotic pressure of blood by about 30%. The secretion showed only 120 mM-K^+—a decrease of 37% below average.

D. *Electrical potentials*

Differences in electrical potential across the gland wall were easily measured by exploiting the secretion itself to establish electrical contact with the lumen. One electrode was simply positioned in the drop of fluid emerging from the gland aperture; the other was inserted into the thoracic haemocoel. This procedure permitted detection of consistent potentials which decreased only as the secretion rate also diminished. With respect to haemolymph the lumen was always positive by approximately 25 mV. With four physostigmine-stimulated animals, the following values were obtained: 28 mV. decreasing to 22; 33 to 20; 23 to 19; and 24 to 8.

The following experiment suggests that this potential was caused, not by ionic differences between secretion and blood, but by an active process of ion transport.

The potential was first measured as usual, and found to be +25 mV., lumen positive. A drop of secretion was then collected from the same animal and placed on a piece of dialysis membrane, which in turn was positioned on a drop of haemolymph collected from the moth. The transmembrane potential was immediately measured and found to be −18 mV. In other words, the secretion was *negative* with respect to blood when the potential merely reflected passive flow of ions across a dialysis membrane.

E. *Effects of inhibitors*

Rigorous evaluation of inhibitor effects must await isolated *in vitro* preparations, since the gland is greatly affected by the condition of the animal as a whole (section A). However, preliminary information has been collected by *in vivo* experiments.

Two specific inhibitors of active transport were tested: acetazoleamide ('Diamox'), an inhibitor of carbonic anhydrase, and ouabain, an inhibitor of Na^+–K^+ exchange. In final concentrations as high as 8×10^{-4} M, Diamox had no detectable effect on the rate of secretion, even when administered one day prior to assay. Ouabain had no effect at a final concentration of 2×10^{-4} M, but it decreased the secretion rate by half at 5×10^{-4} M, and by two-thirds at 8×10^{-4} M. It may be noted that these concentrations were much higher than are normally required for direct interference with susceptible active transport processes: 1×10^{-6} M is sufficient for 50% inhibition in the frog skin (Ussing, Kruhøffer, Thaysen & Thorn 1960). Most probably, the effect was indirect and attributable to the pronounced flaccidity of ouabain-treated moths. Diamox injections produced no such visible ill effect.

By contrast, the injection of 2,4-dinitrophenol markedly affected secretion. At a final concentration of 10^{-4} M it depressed the rate to 30% of normal without otherwise affecting the behaviour of the physostigmine-injected moths. At a level of 5×10^{-4} M it completely abolished secretion without inducing more than a transient flaccidity.

CO_2 was also an effective inhibitor; when secreting moths were exposed to a stream of CO_2, secretion of fluid promptly stopped, but was again resumed upon return to air.

DISCUSSION

A. *Role of the gland*

The labial gland of adult saturniids, being derived from a portion of the larval silk-gland (Kafatos & Williams, 1964), is homologous with the labial salivary apparatus of other insects. In a sense, then, the fluid-producing adult gland shows 'reversion' to a function phylogenetically more primitive than its larval function, secretion of silk. Edwards (1964) has advanced the interesting suggestion that the adult gland may, in fact, be involved in the regulation of water balance, as in the case of *Thysanura* and certain Heteroptera. Thus, when challenged with injections of distilled water or isotonic saline (KCl or NaCl), adult *H. cecropia* employ the labial gland to release fluid. However, the gland could not play a major regulatory role; at least in *A. pernyi* it does not modify the osmotic pressure of blood, and could only bring about minor changes in ionic composition. The S/B ratios indicate that secretion of 150 μl. (about 10% of blood volume) could raise the blood concentration of excluded cations by a maximum of 10%. Only in the case of K^+ is the secretory activity of any consequence;

as expected from the value of S/B, secretion of 100 μl. results in a 20–30% depression in the level of blood K^+.

The large volumes of secretion required to bring about even minor changes in blood composition are never spontaneously released by a normal moth, except during escape from the cocoon. Even *H. cecropia* rarely releases more than 40 μl. of fluid after the first day of adult life (Edwards, 1964). In *A. pernyi*, a species of shorter life span, the ability to secrete declines even more rapidly after emergence, with a concomitant morphological deterioration of the secretory cells (Kafatos & Feder, 1968).

Most likely, under natural circumstances, the saturniid gland is concerned only with escape from the cocoon. In species which have completely sealed cocoons the secretion is a necessary component of the escape mechanism, serving as the solvent and buffer for the enzyme, cocoonase. In species like *H. cecropia*, where the cocoons are equipped with a preformed escape valve, cocoonase is lacking and the fluid probably serves a secondary role as a non-specific softener for the dry fibres of the valve (Edwards, 1964).

B. *Control of secretion*

If the animal is removed from the cocoon prior to ecdysis, little or no fluid is released spontaneously. Apparently the normal stimulus for secretion is mechanical confinement, which normally persists within the cocoon even after the splitting of the pupal cuticle. In an elegant experiment Edwards (1964) showed that artificial restraint of the wings and legs with cellulose tape triggers secretion. The mechanisms of stimulation by physostigmine and inhibition by dissection are unknown. Conceivably the rate of secretion is controlled via the nervous system. Vital staining with reduced methylene blue (Larimer & Ashby, 1964) revealed that the proximal end of the gland (near the orifice) is enveloped by a scant supply of fine nerves from the suboesophageal ganglion. No nerves were observed in extensive electron microscopic studies on the more distal main body of the gland; they would be superfluous if the gland cells are electrically coupled (Lowenstein & Kanno, 1964).

C. *Composition of the elaborated fluid*

It is particularly fortunate that only one type of cell is present in the labial gland; consequently, the authentic secretion can be obtained without alteration by extraneous tissues. The cells elaborate a solution isotonic with blood, and with potassium as essentially the only cation; when available, Rb^+ is also secreted, with almost as high efficiency as K^+. By contrast Na^+, Li^+, Sr^{2+}, Ca^{2+} and Mg^{2+} are all excluded from the secretion—the divalent cations, and especially Mg^{2+}, more completely than Na^+ and Li^+. Among the anions, phosphate is efficiently excluded, while chloride is found in concentrations nearly as high as in blood. By far the principal anion is bicarbonate. The sum of K^+, Cl^- and HCO_3^- is consistent with electrical neutrality and with the observed osmotic pressure; consequently, no significant amounts of other small molecular weight solutes can be expected in the fluid.

Initial drops of fluid ('early secretion') are generally similar to later samples, but differ in having H^+, Na^+, Ca^{2+} and Mg^{2+} (and Sr^{2+}) in somewhat higher concentrations, reminiscent of blood. The discrepancy may be explained in terms of a slow diffusion equilibrium, which is allowed to prevail in the 'resting' gland, but which is

not attained during rapid secretion (see below). In support of this interpretation physostigmine-injected moths released a liquid similar to 'early secretion' when allowed to resume secretion following a temporary inhibition by CO_2 or cold.

D. *Mechanism of secretion*

For rigorous studies of tissues suspected of active transport of ions isolated preparations are required (Ussing & Zerahn, 1951). Thus far, the small diameter of the tubule and the inhibition of secretory activity by injury have frustrated attempts at perfusion and *in vitro* studies. However, sufficient data are available from *in vivo* studies to establish certain conclusions with reasonable assurance.

It is estimated that each secretory cell produces a volume of fluid equal to its own volume within about a minute. Thermodynamic considerations exclude the possibility that this prodigious rate of secretion could be based on active transport of water. Thus, only two alternatives remain: active ion transport or ultrafiltration.

At the time of ecdysis appreciable hydrostatic pressure can develop in the haemolymph as a result of contractions of the abdominal intersegmental muscles (Lockshin & Williams, 1965). Similar contractions are elicited by injection of physostigmine. Thus, at first sight, the possibility that secretion is a direct result of high blood pressure might seem reasonable. Yet, blood pressure could only accelerate secretion, by facilitating flow within the narrow lumen of the gland; it could not, in itself, cause the crucial event of secretion—passage of fluid across the cellular wall into the lumen. The cells of the wall lack both the necessary rigidity (as shown by the loss of turgor in excised pieces of tubule) and the ultrastructural porosity required for ultrafiltration. By contrast, they show striking ultrastructural resemblance to actively transporting cells. The unique composition of the fluid, which is deficient not only in most ions but even in uncharged low molecular components of blood, argues against the idea of a passive process. Finally, secretion persists, albeit at a decreased rate, in partially dissected preparations, which cannot develop hydrostatic pressure.

We are thus led to seek among the components of the secretion one or more ions which could be actively accumulated in such a way as to set in motion events culminating in elaboration of the complete fluid. The outstanding candidate is K^+. In the absence of bulk flow based on ultrafiltration K^+ is apparently transported actively against both electrical and concentration gradients. Na^+, Ca^{2+}, Mg^{2+} and Cl^- all move *with* the electrochemical gradient. Like K^+, HCO_3^- appears to accumulate in amounts far in excess of those compatible with passive processes. However, because of the H_2CO_3–HCO_3^- equilibrium, HCO_3^- need not be transported as such across the cell (see below).

Despite the absence of critical *in vitro* evidence a plausible model for secretion can be constructed with the physiological and morphological information at hand. The key process in this model is a K^+–H^+ exchange, analogous to that described in fermenting yeast (Conway & Brady, 1950) and in respiring mitochondria (Pressman, 1965). Emphasis is placed on the morphological specialization of *both* apical and basal regions of the cells (bordering the lumen and haemocoel, respectively). Both regions are exceedingly rich in mitochondria (Kafatos & Feder, 1968), which maintain close proximity to the deeply and extensively plicated plasma membrane. In short, active processes may be presumed to occur at both sites (Fig. 7).

In the basal region vigorous respiration could energize a proton-pump (Lehninger, 1967), either through a high-energy chemical intermediate (Chance, Lee & Mela 1967) or directly through chemiosmotic coupling (Mitchell, 1967). H^+ would be extruded from respiring mitochondria, across the thin zone of perimitochondrial cytoplasm and the plasma membrane, into the extracellular fluid bathing the basal membrane infoldings. In exchange K^+ would move from the extracellular fluid into the mitochondria (Fig. 7, A). On balance the fluid would be acidified and the mitochondria charged with K^+ and OH^-.

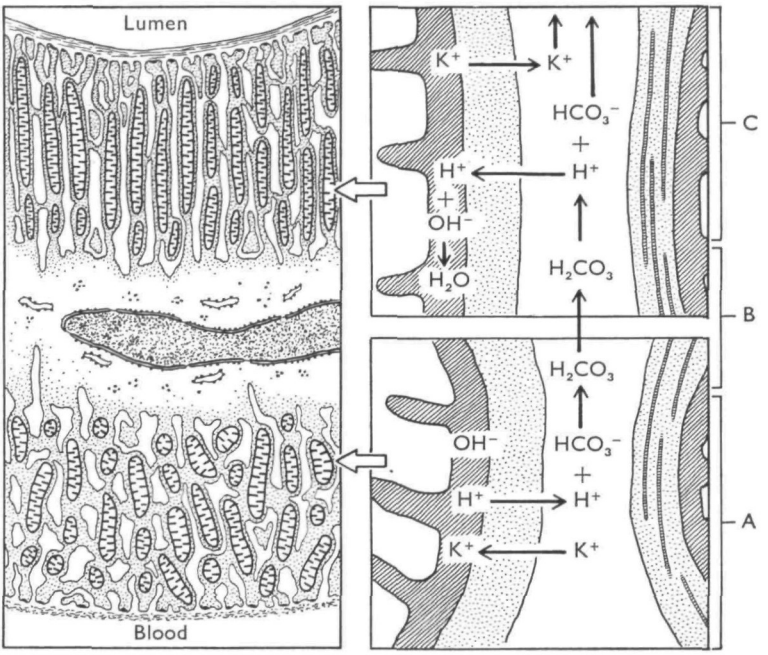


Fig. 7. *Left*: diagram of part of a labial gland cell, showing the mitochondria-rich basal and apical regions, separated by an intermediate zone with the nucleus and other organelles. *Lower right*: higher magnification diagram of the basal region, indicating reactions postulated to occur there. Notice the proximity of mitochondria and plasma membrane, and the occurrence of microtubules (right). *Upper right*: a similar diagram of the apical region. For details, see text.

K^+ and base would now have to be transferred to the apical side of the cell, near the lumen. Though much of the transfer could be accomplished by intramitochondrial diffusion (Anderson & Harvey, 1966), it is tempting to speculate that mitochondrial translocation may also play a role. The cells are characterized by abundant microtubules, which are aligned in the basal-apical axis (Kafatos & Feder, 1968) and often appear anchored to the cell membrane via dense fibrous material, near the tips of both apical and basal cell plications. Microtubules may be involved in cytoplasmic motility (Rudzinska, 1965; Porter, 1966). Thus, the plications might possibly be visualized as transient structures, continuously forming and then reabsorbed into the main body of the cell through the activity of microtubules. Such motion could easily lead to exchange of mitochondria between the basal and apical regions. In any case K^+ reaching the apical zone through diffusion or translocation (Fig. 7, B) could participate

in a reverse exchange with H^+ across the plasma membrane; K^+ and OH^- would be accumulated in the extracellular fluid among the apical plications, and H^+ would return to the mitochondria (Fig. 7, C).

The CO_2-bicarbonate buffer system could offer the basis for stabilization of the pH gradient between blood and secretion. In blood, and even more in the acidified extracellular fluid of the basal region (Fig. 7, A), at least half of the total carbonate would be undissociated (H_2CO_3 or dissolved CO_2). In this form CO_2 is known to diffuse rapidly across cellular membranes (Jacobs, 1940; Chinard, Enns & Nolan, 1960; Fig. 7, B). In the apical extracellular fluid alkalinization would convert H_2CO_3 to HCO_3^-; the latter is not nearly as mobile across membranes. On balance, CO_2 would be trapped into the apical region in a virtual HCO_3^- sink (Fig. 7, C). This would explain the rapid transfer of abundant carbonate from blood into secretion (see Results, § C(3)) without recourse to a separate active transport mechanism.

Accumulation of $KHCO_3$ in the lumen should attract water osmotically from the blood. In this way, the three major components of the secretion, K^+, HCO_3^- and H_2O would be assembled. Other ions would also move toward the lumen, passively following the electrochemical gradient. If the secretion were to accumulate, these ions might be expected to attain a passive equilibrium in which, as can be deduced from the Nernst equation,

$$\log \frac{S}{B} = \frac{XE}{59} \qquad (1)$$

where: E = transtubular potential (lumen positive; mV.), X = negative valence (-1 for a monovalent cation and $+1$ for a monovalent anion). Thus, at equilibrium, the secretion would not be drastically different from blood in composition—in agreement with the presumed composition of 'stored' fluid. However, very asymmetric distribution could be attained upon stimulation of the glands; because of rapid fluid removal concentrations of passively moving ions (Cl^-, Na^+, Ca^{2+}, Mg^{2+}) would reflect a steady-state rather than an equilibrium. S/B would be determined not only by the electrical gradient but also by the permeability of the cells to the corresponding ion and the rate of secretion. The higher the permeability, the greater S/B would be up to the limiting value given by equation (1). The actual values of S/B indicate that the secretory cells are appreciably permeable to Cl^-, slowly permeable to Na^+, and rather impermeable to Ca^{2+} and Mg^{2+}. This order of permeabilities is the same as in muscle fibres (Conway, 1954); the parallelism adds weight to the assumption that back-diffusion of HCO_3^- from secretion to blood is limited, since HCO_3^- enters into muscle at a net rate of only 1% that of Cl^- (Conway, 1954). Most probably cation permeability is determined by the diameter of the hydrated ion (Na^+ = 11·2, Ca^{2+} = 19·2, Mg^{2+} = 21·6 Å.; Conway, 1954). However, the postulated active step, H^+–K^+ exchange, demonstrates additional specificity, since K^+ (hydrated diameter = 7·6 Å.) is preferred over the smaller Rb^+ ion (7·2 Å.).

An attractive feature of this model is that it accounts for the rapid and prolonged release of a $KHCO_3$-rich secretion, without invoking unprecedented transport mechanisms, and without postulating any net ionic changes in the cell (e.g. acidification or Na^+-depletion). By contrast, if each K^+ ion were obtained in exchange for a Na^+ originally contained in the cell, 1 mg of Na^+ (30% of the wet weight of the gland) would have to be expended for the production of 250 μl of fluid.

E. *Comparison with other systems*

An active potassium transport mechanism, linked to electron transport, is known to operate in yeast (Conway, Brady & Carton, 1950); as in the labial gland, K^+ accumulation is ouabain-insensitive. Under certain conditions HCO_3^- appears within the fermenting cells in amounts equivalent to the K^+ accumulated, while an equal amount of H^+ is released into the medium (Conway & Brady, 1950). The analogy between labial gland and yeast is extensive, except that in yeast $KHCO_3$ is retained within the protoplast, whereas in the gland it is transported across the cell and sequestered into the lumen.

A process somewhat similar to that postulated for the labial gland, but in the opposite direction, is thought to occur in the distal tubule of the vertebrate kidney (Davson & Eggleton, 1962). Na^+ is absorbed from the tubular fluid (here analogous to *haemolymph*) in exchange for cellular H^+; the fluid is thus acidified, its HCO_3^- is converted to H_2CO_3, and the latter diffuses back across the cells into the blood (here analogous to the labial *secretion*).

Potassium secretion is well known in insects (Shaw & Stobbart, 1963). In a series of elegant papers, Ramsay (1953–56) documented the central role of K^+ in the elaboration of urine by Malpighian tubules of a number of insects. Apparently, the prime event is the active transport of K^+ into the lumen; Na^+, Ca^{2+} and Mg^{2+} follow passively, at least in the stick-insect, although the Na^+ concentration in urine may occasionally be sufficiently high to indicate active transport. Cl^- is excreted at a concentration slightly lower than in blood, but phosphate is somewhat accumulated; both anions move with the electrochemical gradient. As in the labial gland, the secretion is more alkaline than blood. The transtubular potential is approximately +21 mV., lumen positive (Ramsay, 1953). Clearly, appreciable similarities exist between diuresis and secretion by the labial gland. Recent success in maintaining active Malpighian tubules in synthetic media (Berridge, 1966) promises major advances in the study of this interesting system.

With rigorous *in vitro* methods Harvey & Nedergaard (1964) demonstrated that the larval midgut of *H. cecropia* transports K^+ from the blood into the positively charged lumen, in an active process which does not require Na^+ but may well involve H^+ or HCO_3^-. Just as with the labial gland (Kafatos, 1968; Kafatos & Feder, 1968), ultrastructural studies on the midgut suggest intimate and direct involvement of mitochondria in the transport of K^+ (Anderson & Harvey, 1966). In the midgut system ouabain is ineffective, whereas inhibitors of carbonic anhydrase (at moderately high concentration) or dinitrophenol depress secretion (Haskell, Clemons & Harvey, 1965). Anderson & Harvey (1966) have advanced the intriguing hypothesis that two fundamentally different ion transport mechanisms can be distinguished: (1) the classical, ouabain-sensitive Na^+–K^+ exchange, probably based on the plasma membrane; and (2) an ouabain-insensitive mechanism, associated with mitochondria. In several cases the latter seems to result in separation of acid from base; it may well involve exchange with H^+.

Another potassium-secreting tissue in insects is the epithelial sheet of the testis in *H. cecropia* (Michejda & Thiers, 1963). During diapause, the testicular fluid is essentially in ionic equilibrium with blood. Later on, during adult development, it is

enriched in K^+, at the expense of other cations. As with the labial gland, injections of either Na^+, K^+ or sucrose lead to further accumulation of K^+.

It appears likely that Anderson & Harvey's second mechanism, centred around a H^+–K^+ exchange, may be of fundamental importance in the physiology of insects. Phytophagous species, as a result of their diet, are challenged with an over-abundance of K^+ and a paucity of Na^+. Thus they may be forced to orient many secretory processes around K^+, rather than Na^+. It is noteworthy that in a phytophagous vertebrate, the lizard *Iguana iguana*, K^+ and HCO_3^- are the principal ions of the nasal secretion (Schmidt–Nielsen, Borut, Lee & Crawford, 1963).

SUMMARY

1. The labial gland of adult saturniids produces a voluminous liquid at about the time of ecdysis, in response to either confinement of the moth or physostigmine injection. This liquid normally facilitates escape from the cocoon.

2. During secretion the gland accumulates K^+ and HCO_3^- from the blood, and Rb^+ if it is available, but excludes rather efficiently Na^+, Li^+, Sr^{2+}, Ca^{2+}, Mg^{2+}, phosphate and other small molecular weight components of blood. Cl^- is approximately equally distributed in blood and secretion.

3. The pH of the secretion is approximately 8·5, as compared with 6·5 for blood.

4. The secretion is in osmotic equilibrium with blood, even after alteration of blood osmotic pressure by injection of hypertonic or hypotonic solutions.

5. For both Cl^- and Na^+ the rate of entry into the secretion is proportional to concentration in blood; i.e. the efficiency of exclusion is characteristic for each ion, within a wide range of blood concentrations.

6. Secretion is accompanied by a potential difference across the secretory cell (*ca.* +25 mV. lumen positive). Secretion is abolished by dinitrophenol, but not by ouabain or acetazoleimide.

7. The results are best explained in terms of a respiration-mediated active accumulation of blood K^+, in exchange for H^+, in the basal region of the cells; this is presumably followed by release of the K^+ into the lumen, with concomitant reabsorption of H^+ from the secretion. The alkaline secretion then traps blood CO_2 by converting it to HCO_3^-. Water follows osmotically, and other ions enter passively, at a rate determined by the corresponding permeability of the cells.

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