

Active ion transport and β -ecdysone induced differentiation of *Drosophila* *melanogaster* imaginal discs cultured *in vitro*

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SUMMARY

The theory that β -ecdysone initiates developmental changes during insect metamorphosis by causing an increase in intranuclear levels of potassium, together with a concomitant decrease in sodium levels, has been investigated by two methods. First, imaginal discs from late third instar larvae have been cultured with 0.2 $\mu\text{g/ml}$ of β -ecdysone together with inhibitors of active ion transport. Non-specific inhibitors, which may have general effects on sulphhydryl groups, such as iodoacetic acid, *N*-ethylmaleimide, ethacrinic acid and furosemide, inhibit both eversion and differentiation at concentrations of from 10^{-3} M to 2×10^{-3} M. Ouabain, the only specific inhibitor of the active transport of Na^+ and K^+ across membranes, had no effect on development even at a concentration of 10^{-2} M. Second, a medium containing raised levels of K^+ , and reduced concentrations of Na^+ , neither initiated disc development in the absence of β -ecdysone, nor stimulated development induced by suboptimal levels (0.02 $\mu\text{g/ml}$) of β -ecdysone, either in the presence or absence of ouabain. These results suggest that β -ecdysone induced morphogenesis is not dependent upon Na^+ and K^+ concentrations, or on the activity of an ouabain-sensitive ion pump.

INTRODUCTION

Despite the intensive work performed on insect hormones during recent years, little information has emerged concerning their mode of action. In the case of ecdysone, two models have been proposed. One, suggested by Karlson and co-workers, assumes that the direct action of ecdysone at the chromosomal level triggers the synthesis of messenger RNA (see review, Sekeris, 1974), whereas Kroeger and colleagues consider that ecdysone acts via an intranuclear increase in K^+ concentration, and a corresponding decrease in Na^+ levels (see review, Kroeger & Lezzi, 1966).

Several attempts have been made to test Karlson's model by observing the distribution of radiolabelled ecdysone in target cells (Emmerich, 1969; Thomson, Rogers, Gunson & Horn, 1970). However, no clear binding pattern has emerged from these studies, and further experiments are required before firm conclusions can be made.

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Evidence for Kroeger's model relies largely on the incubation of salivary glands and isolated salivary gland nuclei of *Chironomus thummi* in solutions containing varying Na^+ and K^+ ratios. Larval patterns of puff activity are found in the polytene chromosomes when high levels of Na^+ are present, whereas high K^+ concentrations induce a prepupal puffing pattern (Kroeger, 1963). Similar effects have been reported with isolated chromosomes (Lezzi & Gilbert, 1970). Also, cytoplasmic and nuclear levels of Mg^{2+} and Na^+ fall during development, whereas the K^+ concentration rises at the last larval moult, and again at pupation (Kroeger, Trösch & Müller, 1973). However, Clever (1965), in experiments with salivary glands of *Chironomus tentans*, could find no specific differences in the puffing response to KCl and NaCl of equal molarity, and attributed the effects of both to non-physiological culture conditions.

It is apparent that neither theory of ecdysone action can be accepted at present, and fuller investigations are required to establish the primary effect of the moulting hormone. The developmental responses of cultured imaginal discs, one of the principal target organs of ecdysone, would appear to be a suitable experimental system, especially for the investigation of Kroeger's ion-balance model. If this model is valid, the most probable mechanism for its mode of action would be by an alteration of the activity of the sodium pump, the energy for which is supplied by a membrane-bound Na^+ , K^+ -ATPase. Two methods of analysis have been used to test this proposition. Firstly, the effect of inhibitors of active ion transport, and secondly, the reaction of discs to incubation in culture medium with altered levels of Na^+ and K^+ , have been examined.

The inhibitors of Na^+ , K^+ -ATPase used may be divided into three groups: (1) a specific inhibitor, (2) diuretics, and (3) general sulphhydryl group inhibitors. Their general properties are listed below, and their specificities are compared in the discussion section.

(1) Ouabain, a cardiac glycoside, has been found to specifically inhibit Na^+ , K^+ -ATPase in all systems examined (Schwartz, Lindenmayer & Allen, 1975). Most work on this inhibitor has been performed on mammalian tissues, but ouabain-sensitive active ion transport has also been found in amphibia, fish, and in a range of invertebrates, including insects. For example, the Na^+ and K^+ concentrations in the haemolymph of *Drosophila hydei* are significantly altered by the injection of ouabain into the body cavity (Weber-von Grothuss, Hevert, Atzbacher & Wessing, 1974). The minimum level of ouabain required for inhibition varies considerably according to the tissue examined, and ranges from 10^{-9} M to 10^{-3} M (Glynn, 1964).

(2) Ethacrinic acid is one of a class of aryloxyacetic acid derivatives synthesized in order to produce more effective diuretics than the organic mercurials previously available (Beyer, Baer, Michaelson & Russo, 1965). Since the work of Duggan & Noll (1965), tests have shown that ethacrinic acid could inhibit Na^+ , K^+ -ATPase, and it has been suggested that this inhibition might be its mode of action. Furosemide is another diuretic, which nevertheless differs from ethacrinic acid in some properties. Hook & Williamson (1965) showed that furose-

imide is a potent diuretic in man, dog and rat, whereas ethacrinic acid exerts this effect in man only. Both agents were found to inhibit Na^+ , K^+ -ATPase after *in vivo* infusion into the rat kidney.

(3) The inhibitory effect of sulphhydryl reagents on Na^+ , K^+ -ATPase has been well known since Skou (1963) demonstrated inhibition by both *N*-ethylmaleimide and *p*-chloromercuribenzoate, apparently by blocking ATP hydrolysis. These studies were expanded to show that other sulphhydryl inhibitors such as *p*-chloromercuriphenylsulphonate (Fahn, Hurley, Koval & Albers, 1966) and iodoacetic acid (Bowman, Dolgin & Coulson, 1973), also block the sodium pump. Of these, the effect of *N*-ethylmaleimide and iodoacetic acid on imaginal disc development have been investigated.

METHODS

The procedures used for the sterile culture of larvae from an Oregon S stock of *Drosophila melanogaster*, and for the dissection and culture *in vitro* of their imaginal discs has been previously described (Milner & Sang, 1974). As before, prothoracic leg and wing discs were used. The former were cultured in pairs in order to avoid damage during separation, one pair being considered as a single culture for the purposes of the enumeration of results. Wing discs were cultured and recorded individually. The gift of a sample of *β*-ecdysone from Dr G. B. Russell is gratefully acknowledged. This was isolated from the bark of the tree *Dacrydium intermedium* (Russell, Fenemore, Horn & Middleton, 1972), and contained a minor contaminant of Makisterone A.

The culture medium used was Shields and Sang's medium 3 (Shields & Sang, 1976).

Ouabain was purchased from Sigma, and we gratefully acknowledge the gift of ethacrinic acid and furosemide by Merck, Sharpe and Dohme, and Hoechst Pharmaceuticals, respectively.

RESULTS

The effects of the inhibitors tested, when present throughout the period of culture, were examined at different concentrations. In all cases, a level of *β*-ecdysone (0.2 $\mu\text{g}/\text{ml}$) previously found to be optimal for eversion, separation of the pupal cuticle (apolysis) and the differentiation of imaginal cuticular structures such as bristles, claws and sensilla (Milner & Sang, 1974) was also present.

As before, a relatively subjective presentation of results has been found to be necessary, the degree of development attained being assessed as A, B or C. The symbol A refers to the optimal differentiation of leg or wing discs cultured *in vitro* with 0.2 $\mu\text{g}/\text{ml}$ of *β*-ecdysone, eversion occurring within 24 h, apolysis within 48 h, and the differentiation of specific patterns of imaginal cuticular structures within 72 h. Uneverted leg and wing discs are illustrated in Fig. 1, and their subsequent imaginal differentiation in Fig. 2. C indicates that no eversion

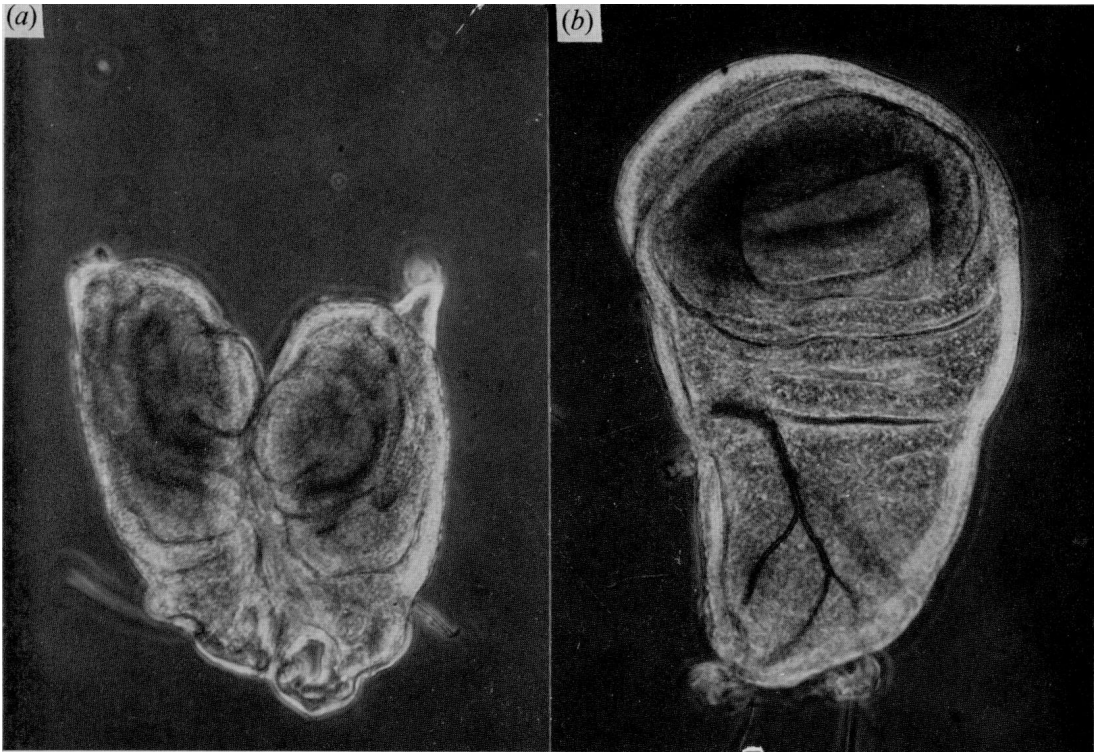
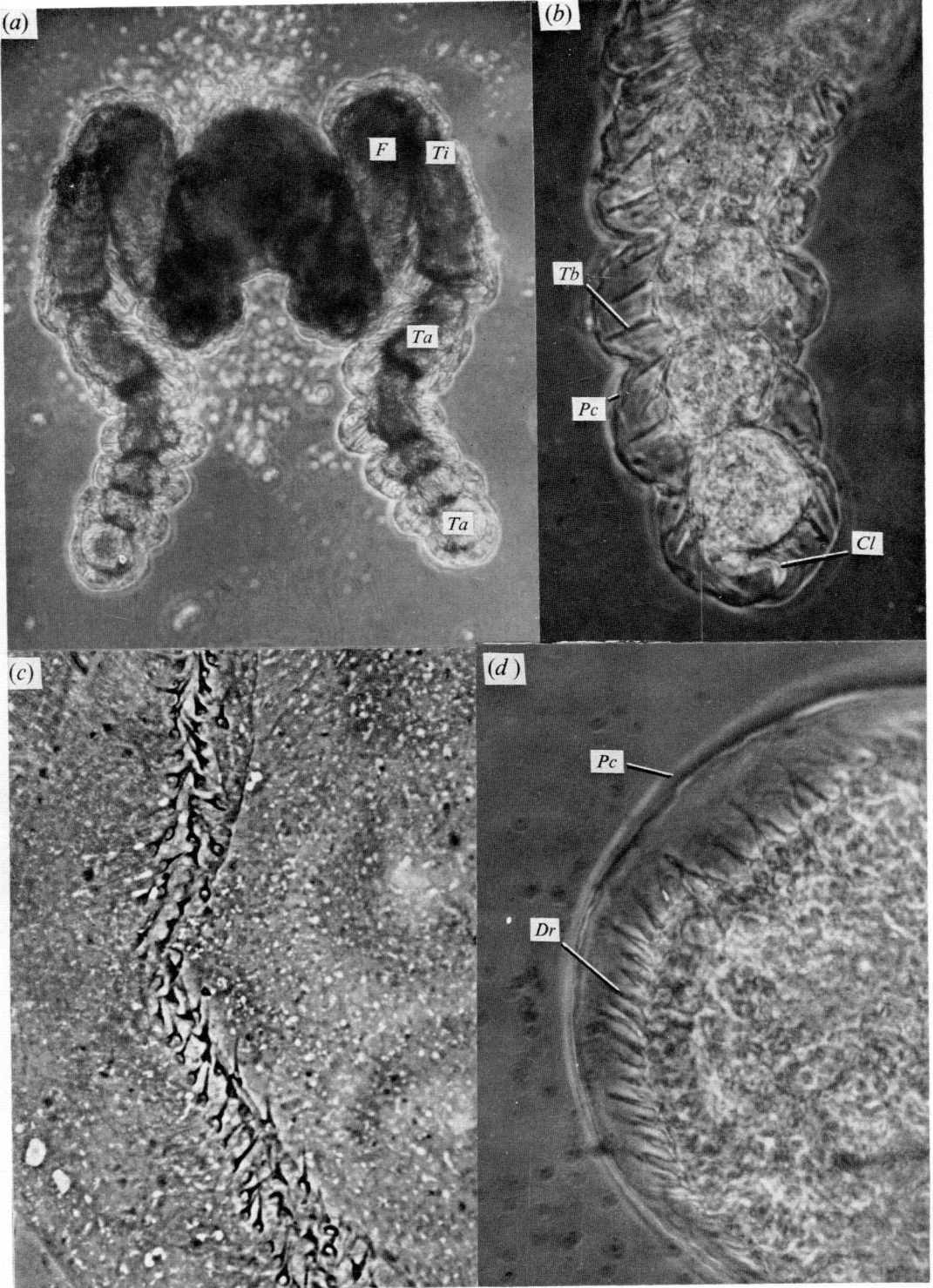


Fig. 1. (a) A pair of prothoracic leg discs, and (b) a wing disc, dissected from a late third instar larva, shortly before pupariation. Both $\times 160$.

or differentiation has taken place, despite the presence of β -ecdysone. B refers to intermediate levels, indicating incomplete eversion and partial apolysis and imaginal differentiation, similar to that found with suboptimal concentrations of β -ecdysone (Fig. 3*b*). When an experiment is designated as class B or C, all the discs examined conformed to the given classification. Because of abnormalities in classes B and C induced by the presence of inhibitor, especially a high level of cell necrosis, exact correlations with the eversion and differentiation index previously used (Milner & Sang, 1974) was not possible. However, class A is equivalent to point 4 on this index. Nearly all the discs in this class differentiated imaginal structures after 3 days in culture (93% and 97% for leg and wing

Fig. 2. The differentiation of leg and wing discs cultured with an optimal concentration (0.2 $\mu\text{g/ml}$) of β -ecdysone. (a) A pair of leg discs, fully everted and differentiated, showing femur (*F*), tibia (*Ti*), and the five tarsal segments (*Ta*). $\times 150$. (b) Tarsal differentiation, showing the pupal cuticle (*Pc*) separated from the underlying tissue during apolysis, and the tarsal bristles (*Tb*) and claws (*Cl*) subsequently differentiated. $\times 420$. (c) A squash preparation, showing triple row bristles differentiated along the anterior margin of the everted wing blade. $\times 450$. (d) The differentiation of the proximal part of the wing; bristles of the double row (*Dr*) beneath apolysed pupal cuticle (*Pc*). $\times 415$.



discs respectively). Almost all wing discs everted, whereas a fairly constant proportion (about 50 %) of leg disc pairs did not undergo this process normally. Here, the tarsi everted within the peripodial membrane up to stage 4 as described by Chihara, Petri, Fristrom & King (1972). The peripodial membrane, instead of breaking down, contracted back to reform the original shape of the disc, the everted tarsi remaining within the disc, and undergoing apolysis and imaginal differentiation. Specific patterns of bristles, hairs and/or sensilla from other regions of the leg such as the tibia, femur, trochanter and coxa may be seen in the differentiated tissues of internally everted pairs of leg discs. The original classification of these discs as merely showing 'internal rearrangement which did not result in eversion' (Milner & Sang, 1974) was an underestimate of their developmental potential, as only eversion, and not differentiation, was affected. Thus, the classification A for cultures of prothoracic leg discs indicates that the usual proportions of discs underwent eversion or internal eversion, and that differentiation subsequently occurred in both classes.

In most of the experiments described below, wing discs were used, because of the greater uniformity of their response to β -ecdysone. However, pairs of leg discs were used in some of the inhibitor studies, to determine whether the frequency of internal eversion was affected by the addition of ion pump inhibitors. In no case was such an effect observed. Leg and wing discs show similar levels of developmental response to β -ecdysone *in vitro* over the range of concentrations tested (Milner, 1975).

The effects of a range of concentrations of ouabain, ethacrinic acid and furosemide on the responses of discs cultured with optimal levels of β -ecdysone are summarized in Table 1. Sulphydryl group inhibitors *N*-ethylmaleimide and iodoacetic acid were tested at 10^{-3} M, a concentration previously found to be inhibitory in other systems (Webb, 1966). It can be seen that ethacrinic acid, *N*-ethylmaleimide and iodoacetic acid completely inhibited disc eversion at 10^{-3} M, a slightly higher concentration of furosemide (2×10^{-3} M) being required for a similar effect. In contrast, concentrations of ouabain as high as 10^{-2} M, approaching saturation levels for this compound, did not inhibit the normal sequence of development in any way.

The second approach used to investigate the involvement of altered ion ratios in disc development utilized the culture of discs in medium with altered concentrations of Na^+ and K^+ . For this, an experimental medium was prepared in which sodium glutamate and sodium dihydrogen orthophosphate were replaced by their equivalent potassium salts. This reduced the Na^+ concentration to 25% of normal, the remaining Na^+ coming solely from the serum additive (G. Shields, unpublished observations). The K^+ concentration was increased from 48 mM to 103 mM, thus maintaining the normal overall concentration of ions in the medium. The control medium batch had the usual Na^+ and K^+ concentrations of 73 mM and 48 mM, respectively.

Imaginal discs were cultured without β -ecdysone, to determine whether the experimental medium alone would stimulate eversion and differentiation.

Table 1. The effect of different inhibitor concentrations on the developmental response of leg (L) and wing (W) discs to 0.2 μ g/ml of β -ecdysone

Concentration of inhibitor	0		10 ⁻⁵ M		10 ⁻⁴ M		10 ⁻³ M		2 × 10 ⁻³ M		10 ⁻² M	
	L	W	L	W	L	W	L	W	L	W	L	W
Ouabain	A (25)	A (20)	—	—	—	—	A (40)	A (21)	A (19)	—	A (20)	—
Furosemide	A (20)	A (10)	—	A (20)	—	—	B (20)	—	C (15)	C (15)	—	—
Ethacrinic acid	A (25)	A (12)	—	A (20)	—	B (20)	C (25)	C (15)	—	—	—	—
N-ethylmaleimide	—	A (20)	—	—	—	—	—	C (20)	—	—	—	—
Iodoacetic acid	—	A (15)	—	—	—	—	—	C (15)	—	—	—	—

A. The normal sequence of eversion and differentiation *in vitro* with an optimal β -ecdysone concentration.
 B. A lower level of response; partial eversion, poor cuticle separation and imaginal differentiation.
 C. No eversion or differentiation, perhaps some necrosis. Numbers in parentheses refer to the number of wing discs, or leg disc pairs, examined.

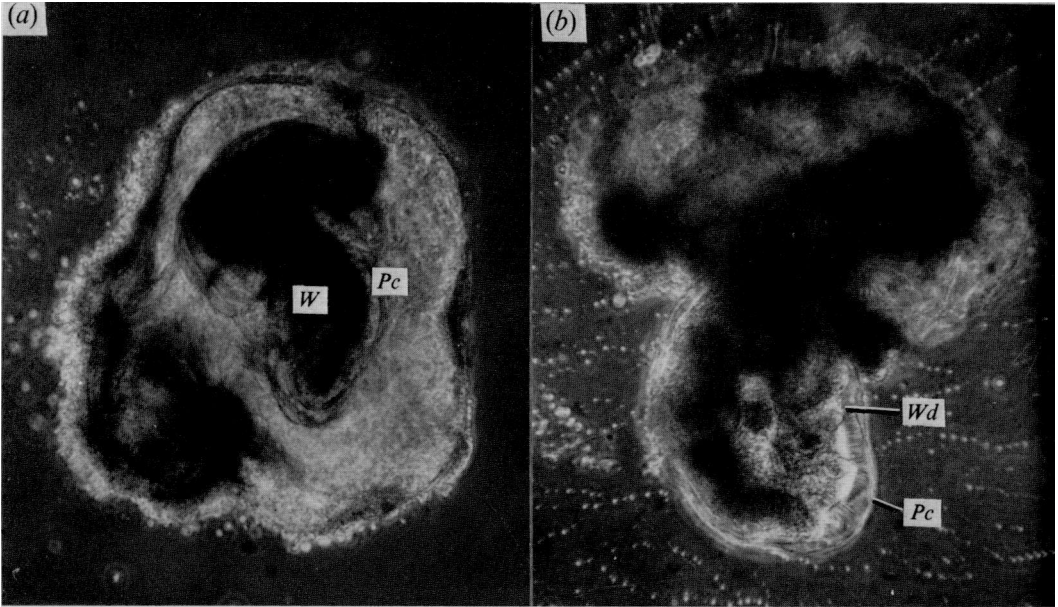


Fig. 3. The development of wing discs cultured with a suboptimal concentration ($0.02 \mu\text{g/ml}$) of β -ecdysone. (a) The distal part of the wing blade (*W*), internally everted inside a hollow vesicle. Separation of the pupal cuticle (*Pc*) has occurred. (b) An incompletely everted wing blade. Apolysed pupal cuticle (*Pc*) and wing differentiation (*Wd*) can be seen. Both $\times 140$.

Twenty wing discs were cultured individually for 6 days in each of the two media, but there was no initiation of development by the experimental medium. However, it might be argued that an active ion pump mechanism could maintain the initial Na^+/K^+ balance against a slow diffusion of ions through the cell membrane. The experiment was therefore repeated using media containing a high level (10^{-3} M) of ouabain. As before, there was no difference between control and experimental series: neither the eversion of appendages nor the differentiation of imaginal structures occurred in any of these cultures. However, approximately 15% of discs in both control and experimental cultures underwent a very low level of development, probably due to an *in situ* exposure to ecdysone before dissection.

Although the high $\text{K}^+/\text{low Na}^+$ medium did not initiate development in the absence of β -ecdysone, it seemed possible that this ionic environment might stimulate the response of discs to a suboptimal concentration of β -ecdysone ($0.02 \mu\text{g/ml}$, see Milner & Sang, 1974). With this level of moulting hormone, wing discs frequently underwent a process of internal eversion similar to that described above for leg discs cultured with an optimal concentration of β -ecdysone ($0.2 \mu\text{g/ml}$). After partial eversion, a re-contraction of the peripodial membrane resulted in the formation of a hollow vesicle, enclosing the partially everted wing blade, which might subsequently undergo apolysis and imaginal differentiation (Fig. 3a). Alternatively, the peripodial membrane might break

Table 2. *The developmental responses of wing discs in medium containing low levels of Na⁺, and high levels of K⁺*

Medium	No. of wing discs	Days after culture initiation	Apolysis (%)	Differentiation of imaginal cuticular structures (%)
Control	30	2	70	0
		3	97	7
		4	97	50
		5	97	53
Experimental	30	2	77	0
		3	100	10
		4	100	40
		5	100	40
Control + ouabain 10 ⁻³ M	30	2	83	0
		3	97	0
		4	97	13
		6	97	27
Experimental + ouabain 10 ⁻³ M	30	2	90	0
		3	100	7
		4	100	20
		6	100	43
		8	100	67

down, leaving the wing blade partially everted (Fig. 3*b*), the degree of eversion attained being very variable. Apolysis and imaginal differentiation occurred at a slower rate than with optimal ecdysone levels, and were frequently not found in all cultures. Because of the difficulty of categorizing different degrees of development, the percentage of wing discs showing any apolysis or imaginal differentiation, whether extensive or restricted, was noted on several days during the development of the cultures. It is suggested that this may provide a reasonable basis for the comparison of rates of development.

As before, wing discs were cultured in control and experimental media, both with and without 10⁻³ M ouabain (Table 2). It is apparent that the levels of development initiated in both media by 0.02 μg/ml of β-ecdysone were similar, irrespective of the presence or absence of ouabain. Without the ion transport inhibitor, a slightly higher level of differentiation was attained in control than in experimental cultures, whereas the reverse was the case in cultures containing ouabain. However, these differences were not significant using 2 × 2 contingency tables, and were within the range of variation expected with this suboptimal concentration of β-ecdysone. The rate of development appears to be slower in medium containing ouabain than in medium lacking ouabain. However, the former cultures were initiated 3 weeks later than the latter cultures, and during this time a slight deterioration in the culture medium in terms of the rapidity of

disc development, would be expected (Milner, 1975). Also, the qualitative appearance of the cultures in terms of the final levels of eversion, apolysis and degree of differentiation was similar in all four cases. Thus, increasing K^+ and decreasing Na^+ concentrations in the culture medium did not appear to stimulate development.

DISCUSSION

In intact membrane preparations, ouabain inhibits the sodium pump only when present in the milieu bathing the preparation, suggesting that the receptor for the drug resides on the external surface of the cell membrane. Also, [3H]-ouabain specifically binds to receptor sites on membrane fragments associated with the sodium pump. Indeed, the site of action of the drug is very highly correlated with Na^+ , K^+ -ATPase, since the most highly purified preparations of this enzyme, possessing only two major polypeptides, retain their sensitivity to ouabain. It would appear that inhibition by ouabain is an allosteric phenomenon, involving alterations in the conformation of the pump, since the receptor for the drug appears to lie on the external surface of the cell membrane, whereas the active site for the ATPase resides on the internal surface. In spite of intensive work, however, the exact mechanism of action remains unclear (for extensive review see Schwartz *et al.* 1975).

A similar high level of specificity is not found for diuretic agents which inhibit Na^+ , K^+ -ATPase, such as ethacrinic acid and furosemide. Ethacrinic acid inhibited respiration and glycolysis in both intact tissues (Epstein, 1972), and in cell-free systems, where secondary effects of the inhibition of ion transport would not be expected to occur (Klahr, Yates & Bourgoignie, 1971). Furosemide has also been found to inhibit respiration (Yoshida & Metcalf, 1970), but whether by the same mechanism as ethacrinic acid remains unclear.

Whereas some authors consider that ethacrinic acid may act by binding sulphhydryl groups, known to be required for Na^+ , K^+ -ATPase activity (Duggan & Noll, 1965; Davis, 1970), others suggest that this interaction is not the major source of the diuretic effects of the drug (Epstein, 1972; Burg & Green, 1973). However, it is apparent that ethacrinic acid and furosemide, unlike ouabain, affect several different areas of cell metabolism in the experimental systems examined, only one of which is active ion transport. Inhibitors which bind sulphhydryl groups, such as *N*-ethylmaleimide and iodoacetic acid, would be expected to have a similarly widespread effect (Webb, 1966). It must be emphasized, however, that little work on ion pump inhibitors has been performed using insect preparations, the above conclusions on the specificity of action being derived for the most part from studies on mammalian renal tissue.

The results presented in Table 1 show that ethacrinic acid, furosemide, *N*-ethylmaleimide and iodoacetic acid inhibit β -ecdysone mediated disc development at concentrations of 10^{-3} M to 2×10^{-3} M. However, ouabain, the only inhibitor thought to be specific to Na^+ , K^+ -ATPase, caused no inhibition of development at concentrations as high as 10^{-2} M. This lack of effect is unlikely

to be due to poor cellular penetration, as the site of action of the drug is on the external surface of the cell membrane. Also, ouabain was found to inhibit *β*-ecdysone-induced vesicular swellings in internally everted imaginal discs cultured with 0.02 $\mu\text{g/ml}$ of *β*-ecdysone (Milner, 1975), demonstrating that this drug can interact with imaginal disc tissue in this experimental system. This strongly suggests that the processes of eversion and differentiation do not require a functional Na^+ , K^+ -ATPase, and that the inhibition of development found with diuretics and sulphhydryl reagents may well be due to disruptions of areas of metabolism unrelated to active ion transport.

These conclusions are supported by experiments using a high K^+ , low Na^+ medium, containing suboptimal levels of *β*-ecdysone (Table 2). No significant differences between the rates of development in control and experimental batches of medium were found, either with or without ouabain, and disc development was not initiated in this medium in the absence of moulting hormone. The levels of Na^+ and K^+ present in the control medium are based on a haemolymph analysis of third instar *Drosophila* larvae (Begg & Cruikshank, 1963), and so should reflect the normal ionic environment of larval tissue shortly before pupariation.

The intranuclear concentration of K^+ in salivary gland cells of *Chironomus thummi* was found to increase by about 15 mM on pupation (Kroeger *et al.* 1973), increases of this magnitude being sufficient to change the puffing patterns of cultured salivary gland chromosomes (Kroeger & Müller, 1973). It was expected, therefore, that doubling the K^+ level in the culture medium, with a concomitant reduction in Na^+ concentrations, would produce a sufficient alteration in K^+ concentrations for differences in development to become apparent over several days of culture, if this change indeed represented the mode of action of ecdysone.

Although it is difficult to disprove a theory using only negative evidence, the above observations, together with the criticisms previously levelled at Kroeger & Lezzi's experiments (for review, see Doane, 1973), appear to provide no basis for supporting the hypothesis that ecdysone acts via alterations in intranuclear sodium and potassium ion concentrations.

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