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Bursicon: Its effect on tyrosine permeation into insect haemocytes

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SUMMARY

When haemolymph of *Pieris brassicae* is incubated in isotonic solutions, tyrosine, a normal constituent of insect haemolymph, is rapidly metabolized with concomitant deterioration of the system. This reaction may be prevented by adding 10^{-4} M sodium diethyldithiocarbamate to the incubates.

In these stabilized haemocyte suspensions as a rule no tyrosine conversion occurs.

If bursicon, the cuticle-hardening hormone, is added to such stabilized cell suspensions tyrosine conversion starts immediately, and proceeds at a rate depending on the amount of bursicon added. Ca^{2+} and Mg^{2+} accelerate this reaction. Since the enzyme system converting tyrosine into DOPA, and the latter to dopamine, has been shown to reside within the haemocytes, whereas the substrate tyrosine occurs in the serum, the above results are considered as evidence in proof of the hypothesis of Mills, R.R. and Whitehead, D.L. (1970) *J. Insect Physiol.* 16, 331–343, that the function of bursicon is to render the haemocyte membrane permeable to tyrosine.

Bursicon has been characterized as an insect hormone inducing hardening and darkening of insect cuticle[1]. It is released from thoracal or abdominal ganglia just after moult or pupal eclosion[1,2]. Mills and Lake[3] purified the hormone following its extraction from newly moulted cockroaches and, in agreement with Fraenkel *et al.*[4], found it to be a polypeptide with a molecular weight of approx. 40 000.

With respect to the mode of action of bursicon, Mills and Whitehead[5] have advanced an interesting hypothesis ascribing to the hormone a regulatory function in the conversion of tyrosine to *N*-acetyldopamine, the principal vulcanizing agent of insect cuticle[6]. In view of earlier observations[7,8] that the enzymes converting tyrosine to DOPA and the latter to dopamine resided within haemocytes, whereas the substrate, tyrosine,

occurred in the serum, Mills and Whitehead[5] suggested that the function of bursicon was to render the haemocyte membrane permeable to tyrosine, thus permitting the latter to enter the cells to be converted into *N*-acetyldopamine. They tried to test this hypothesis by incubating cockroach haemolymph with [U-^{14}C] tyrosine and measuring the uptake of label into the cell fraction after time intervals of up to 60 s. They found that in bursicon-treated haemolymph of intermoult cockroaches the haemocytes did, in fact, take up some 70% more label than did haemocytes in bursicon-free controls. A similarly enhanced uptake was found with haemolymph from newly moulted cockroaches as compared to haemolymph from intermoult ones.

However, if the hypothesis of Mills and Whitehead[5] is valid, it is unlikely that in bursicon-free haemolymph appreciable amounts of tyrosine would be taken up into the haemocytes within such short time intervals. Therefore, the relatively high tyrosine uptake found by these authors in their bursicon-free controls was probably an artifact, and this would somewhat impair the conclusive force of their findings.

We have tried to obtain more substantial proof for this hypothesis by incubating haemolymph for longer time intervals and using the residual tyrosine content of the system as a criterion for its uptake into haemocytes, thereby implying its immediate conversion following entrance. For the tyrosine assay we used the fluorimetric method of Udenfriend[9]. This method does not discern between tyrosine and tyramine. In experiments not recorded here we incubated haemolymph from *Pieris brassicae* (cabbage-white) larvae with L-[*carboxyl*-^{14}C] tyrosine under the conditions specified in the legend to Fig. 1, except that the solution was buffered to pH 5.5, and determined the uptake of label into alkali *via* the gas phase. It was thus found that decarboxylation proceeded at about the same rate as did the decline of the tyrosine concentration, the accumulation of labelled CO_2 lagging 10–20 min behind the disappearance of tyrosine. Hence it seemed plausible that in the cells hydroxylation to DOPA preceded decarboxylation, so that no appreciable tyramine formation occurred. If *Pieris* haemolymph is collected at room temperature it rapidly deteriorates. This process has been associated with the blood-clotting reaction[10]. It manifests itself as a rapid blackening of the haemolymph, caused by melanin formation. Frequently a sequestering agent such as EDTA is used to inhibit blood clotting, and this has also been done by Mills and Whitehead[5] in the work referred to.

We incubated haemolymph from *Pieris brassicae* larvae, diluted 5-fold with isotonic glucose–NaCl solution. If this system contained EDTA in addition, the onset of blackening was postponed by several hours; nevertheless, tyrosine was still decomposed at a fairly rapid rate. In Fig. 1 it is shown that with EDTA concentrations as high as 50 mM the half-life of tyrosine is still little more than 40 min. Paradoxically, with haemolymph from newly moulted caterpillars (not shown) which should contain bursicon, or with bursicon-treated haemolymph from intermoult caterpillars (Fig. 1) practically no tyrosine breakdown occurred.

From these observations the following may be inferred: (a) EDTA, even at 50 mM, is of little use in stabilizing *Pieris* haemocytes, for the tyrosine conversion in the bursicon-free incubates implies its accessibility to the enzyme system inside the cells.

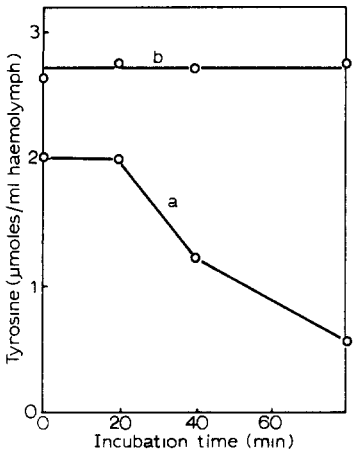
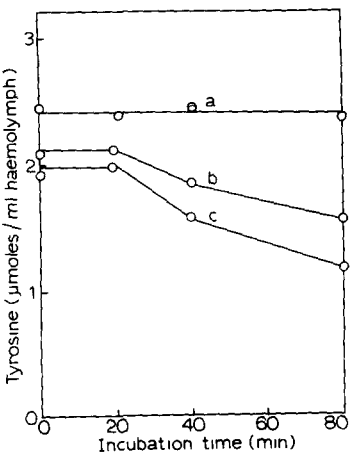


Fig 1. Effect of bursicon on *Pieris brassicae* haemolymph diluted with EDTA–saline, a, without bursicon, and b, with bursicon. Haemolymph (about 400 μl) from five to ten *Pieris* larvae, 2 days after the fourth moult, was collected in 4 times the volume of ice-cold saline (0.2 M glucose, 0 077 M NaCl, 0.05 M EDTA, pH 7 2) In the case of Curve b 5 μl bursicon concentrate were added. Incubation was done at 25 °C At the intervals designated in the graph, 0.1-ml or 0.2-ml samples were taken and assayed for tyrosine according to the method of Udenfriend[9] Bursicon was prepared from house flies (*Musca domestica*) and purified by a procedure similar to that of Mills and Lake[3] The flies were collected in liquid nitrogen between 30 min and 1 h after pupal eclosion For the bursicon test we used the bioassay of Fraenkel and Hsiao[4], using house flies ligatured at the neck The bursicon activity in the preparation used for the work under notice was such that 1 μl injected into the abdomen of a ligaturated fly just caused complete blackening in 20 min.

Microscopic examination revealed that very few cells were actually broken; hence it would appear that tyrosine conversion in the bursicon-free system is due to denaturing of the mechanically intact cell membranes. (b) Bursicon does, in fact, react with the cells, stabilizing them, but it does not effect their uptake of tyrosine in the conditions chosen. Since these conditions imply an amount of EDTA sufficient to sequester all Ca^{2+} and Mg^{2+} present in the incubation system[11], it did not seem altogether unreasonable to suspect this agent of being the cause of the paradoxical bursicon effect. We therefore tried to replace EDTA by a different stabilizing agent.

It appeared likely that the rapid deterioration of freshly diluted haemolymph was initiated by spilling of tyrosinase from a few broken cells, the ensuing conversion of tyrosine to polyphenols causing a cascade effect of membrane deterioration. If this were the case, a tyrosinase inhibitor would cancel the effect and thus function as a stabilizer. But it would also block tyrosine conversion within the cells, thus rendering our system useless, unless the inhibitor failed to permeate into the cells. The ionic tyrosinase inhibitor sodium diethyldithiocarbamate at 10^{-4} M appeared to meet this condition. Fig. 2 shows that in haemolymph of intermoult *Pieris* larvae, diluted 5-fold with isotonic salt solutions, almost no tyrosine conversion occurs if this system contains in addition 10^{-4} M sodium diethyldithiocarbamate. If the inhibitor is omitted in the same



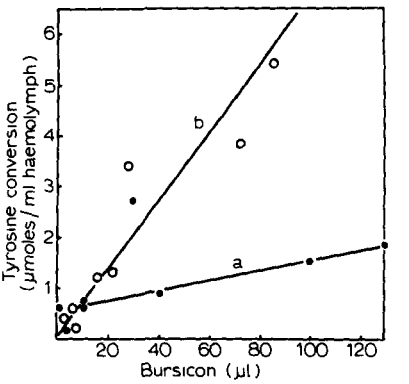


Fig. 2. Effect of bursicon on tyrosine conversion in *Pieris* haemolymph diluted with sodium diethyldithiocarbamate–saline, a, without bursicon, b, with 5 μl, and c, with 20 μl bursicon solution added after 20 min incubation. Haemolymph from *Pieris* larvae was collected 2 days after the fourth moult, in a 4-fold volume of ice-cold salt solution (0.007 M $CaCl_2$, 0.011 M $MgCl_2$, 0.045 M KCl, 0.085 M NaCl, 0 150 M glucose, 10^{-4} M sodium diethyldithiocarbamate). Incubation and sampling as specified in the legend to Fig. 1.

Fig. 3. Tyrosine conversion as a function of the bursicon concentration in two different salt solutions. a, in the medium specified in the legend to Fig. 2; b, in 0.155 M NaCl + 10^{-4} M sodium diethyldithiocarbamate

system some 90% tyrosine decomposition occurs within half an hour. Apparently then the diethyldithiocarbamate in the system functions to stabilize the cells. That it does not inhibit tyrosinase inside the haemocytes may be concluded from the effect of bursicon shown in Fig. 2. Immediately after the addition of the hormone, tyrosine conversion starts, its rate depending on the amount of bursicon added. The latter is shown in more detail in Fig. 3, in which dose–response curves are depicted with two isotonic salt solutions of different compositions. Within the concentration limits investigated the bursicon effect appears to be linear.

If the haemolymph is diluted with solutions containing Ca^{2+} and Mg^{2+} the effect of bursicon is about 3-fold that obtained on dilution with a solution containing Na^+ only. This supports the assumption that Ca^{2+} and/or Mg^{2+} are essential for bursicon to be effective in inducing tyrosine permeation.

The above results suggest that *in vitro* the interaction of bursicon with *Pieris* haemocytes can be demonstrated, provided an ionic tyrosinase inhibitor is added as a stabilizer. Such a system could furnish as an *in vitro* test for bursicon.

A plausible explanation is available for the seeming inconsistency between, on the one hand, the finding by Mills and Whitehead[5] that tyrosine uptake into cockroach blood cells was enhanced in the presence of both bursicon and EDTA and, on the other hand, our results that tyrosine conversion was completely blocked by this combination.

The apparent fact[12] that cockroach haemolymph is far less liable to deterioration than blood cells from other insects enabled Mills and Whitehead to work with 1 mM EDTA in their experiments compared to 50 mM in our work. It is therefore plausible that sufficient free Ca^{2+} and Mg^{2+} remained in their incubations to elicite an effect of bursicon upon tyrosine permeation into haemocytes.

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REFERENCES

1 Fraenkel, G. and Hsiao, C. (1965) *J. Insect Physiol.* 11, 513–556
2 Mills, R.R., Mathur, R.B. and Guerra, A.A. (1965) *J. Insect Physiol.* 77, 1047–1053
3 Mills, R.R. and Lake, C.R (1966) *J Insect Physiol.* 12, 1395–1401
4 Fraenkel, G., Hsiao, C. and Seligman, M. (1966) *Science* 151, 91–93
5 Mills, R.R. and Whitehead, D.L. (1970) *J. Insect Physiol* 16, 331–340.
6 Karlson, P., Sekeris, C.E. and Sekeri, K.E. (1965) *Z. Physiol Chem.* 327, 86–94
7 Mills, R.R., Androuny, S., and Fox, F.R (1968) *J Insect Physiol.* 14, 603–611
8 Whitehead, D.L (1970) *FEBS Lett.* 7, 263–266
9 Udenfriend, S. (1957) in *Methods in Enzymology* (Colowick, S.P. and Kaplan, N.O., eds) Vol. 3, p. 610, Academic Press, London and New York
10 Gregoire, Ch. (1964), in *The Physiology of Insects* (Rockstein, M., ed.) Vol. 3, p. 153, Academic Press, New York and London
11 Florkin, M. and Jeuniaux, Ch. (1964) in *The Physiology of Insects* (Rockstein, M., ed.) Vol. 3, p. 110, Academic Press, London and New York
12 Mills, R.R. and Fox, F.R. (1972) *Insect Biochem.* 2, 23–28

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