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3. The experiments required that the resistance of the surface membrane remain stable for long periods when the cells were impaled with microelectrodes. This condition is rare in epithelial cells; in this regard, experiments with nerve cells have a clear advantage. Of the various epithelia tested, *Chironomus* salivary gland cells provided the best material, and experiments lasting 1 to 2 hours could be run under stable conditions. But even in these relatively sturdy cells, satisfactory results could be obtained only at the expense of considerable number of trials and material.
4. The normal saline solution had the following composition: 87 m*M* NaCl; 2.7 m*M* KCl; 1.3 m*M* $CaCl_2$; 10 m*M* tris buffer. Changes in Ca^{++} concentration and additions of chelators in the test solutions were done by equimolar substitution for NaCl of the normal solution. The molarity of all solutions was thus constant, and the osmolarity constant within 1 percent. The *p*H was 6.3 in all solutions. The Ca^{++} contamination in "Ca^{++}-free" solutions was below 10^{-6}*M*.
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Properties of Bursicon: An Insect Protein Hormone That Controls Cuticular Tanning

Abstract. *On gel filtration the retention volume of bursicon indicates a molecular weight of about 40,000. On disc electrophoresis bursicon migrates toward the anode and appears at an* R_F *(relative to the marker dye) of 0.3 to 0.4. The properties of fractions with bursicon activity in blood, brain, and ganglion of a fly* (Sarcophaga bullata), *and in blood, ganglion, and corpora cardiaca of a roach* (Periplaneta americana) *are similar, but not identical. Bursicon in the blood is more heat labile, and the activity in brain and corpora cardiaca shows two peaks in electrophoresis, instead of one as in the other fractions.*

The action of a hormone which mediates tanning in the cuticle of adult flies has been reported (*1, 2*). At the time of emergence from the puparium the blood is devoid of this hormone. If the head is separated from the body by a ligation on the neck at this time, the rest of the body never tans. Injection of blood from a tanning fly into a ligatured fly causes the latter to tan. The hormone controlling this process is derived from the median neurosecretory cells in the brain and from the abdominal region of the combined thoracico-abdominal ganglion (*3, 4*). This hormone seemed to be entirely different from the conventional insect hormones and was given the name *bursicon* (from the Greek bursikós, pertaining to tanning) (*4*).

Early in the investigation it became clear that bursicon is a protein. It is nondialyzable and is destroyed by alcohol, acetone, several proteases, and trichloroacetic acid (*1, 3, 4*). After precipitation with ammonium sulfate, the activity can be largely recovered.

The following experiments were designed to provide evidence for the protein nature of bursicon. All experiments were carried out with the blowfly *Sarcophaga bullata*, except where stated otherwise.

The methods for detecting and testing for bursicon activity have been described previously (*4*). For a quantitative evaluation of hormone activity, a classification was devised whereby different degrees of tanning were expressed by numbers of plus (+) signs, with three-plus (+++) standing for maximal effect. In instances of suboptimal activity, the abdomen is more intensely tanned than the thorax. Thoraces and abdomina were evaluated separately and the total number of plus signs in all flies in a test was added. (Thus, the maximum value for any fly is six plus signs, three each for thorax and abdomen; and when five flies are used in an experiment, full tanning is expressed by the value 30.)

Blood containing hormone activity was processed through Bio-gel P-200 columns as described (*5*). Samples (100 μl) were placed on columns (14 cm by 0.8 cm), and 20 fractions (0.5 ml each) were collected by elution with 0.005*M* phosphate buffer, *p*H 7.8. The protein content of the fractions was measured with the Folin-Ciocalteu reagent (*6*). The bulk of the blood proteins was excluded from the gel and was contained in fractions 4 and 5, with the amount of protein in subsequent fractions rapidly tapering off. The slight rise in optical density of fractions 11 and 12 was probably due to the presence of polypeptides and amino acids that reacted with the Folin-Ciocalteu reagent (Fig. 1).

In order to get a measure of the molecular weight of bursicon, carbonic anhydrase (mol. wt., 31,000) (Worthington Biochemical Corp.) and hexokinase (mol. wt., 51,000) (Sigma Chemical Co.), were added to blood already containing the hormone activity and processed through the column. The retention value for bursicon and each enzyme was determined. Carbonic anhydrase was measured potentiometrically according to March and Anderson (*7*), and hexokinase according to Andrews (*8*). Maximum bursicon activity was found in fractions 6–8, with the peak almost certainly in fraction 7 (Fig. 1). Tests at higher dilutions of the active fractions, which would have permitted us to determine differences of activity in these three fractions, were omitted. A single peak was obtained in other experiments. Hexokinase showed a peak in fraction 6, and carbonic anhydrase in fractions 8 and 9. The experiment was repeated several times, with a very slight shifting of peaks of activity on different occasions, but the retention volume of bursicon was always roughly intermediate between that of the two enzymes. From this it is concluded that the molecular weight of bursicon is of the order of 40,000.

Active and inactive blood (5-μl sample) was submitted to polyacrylamide disc electrophoresis for acidic proteins,

Table 1. Electrophoretic separation of bursicon activity from various tissues of *Sarcophaga bullata* and *Periplaneta americana*, at *p*H 8.5. Fraction 1 is the fastest moving fraction right behind the marker dye.

Preparation	Bursicon activity of fractions 1 to 11										
	1	2	3	4	5	6	7	8	9	10	11
	Sarcophaga										
Active blood *	3	3	4	1	3	28	25	8	2	3	3
Inactive blood †				1	0	4	4	1	1		
MNC	22	5	5	6	4	5	6	12	10	1	
'Abdominal' ganglion	7	5	6	3	2	19	18	4	6	5	
	Periplaneta										
Corpora cardiaca	18	6	4	1	5	18	20	19	6	2	
Last abdominal ganglion	2	5	3	7	12	18	13	8	5	2	

* From 30-minute-old fly, containing bursicon. † From fly less than 1 minute old, with no bursicon activity.

at *p*H 8.5 (*9*), on columns measuring 5.5 by 82 mm. About 16 bands of varying width and intensity were discerned (Figs. 2*A* and 3*A*), but there was no visible difference between the band patterns whether the blood contained activity or not. The position of bursicon in relation to this band pattern was determined by the following method: Eight columns were run at the same time, each containing 25 μl of blood. After electrophoresis each column was cut into 11 equal portions, by inserting the column into a tube of plexiglass that contained ten equidistant slits through which the column was sliced with a razor blade. Corresponding fractions from the eight columns were pooled, cut into small pieces, and left standing overnight in 1 ml of 20 percent Ringer solution. The eluates were then concentrated in a dialysis bag with the aid of Aquacide (Calbiochem) and tested for bursicon activity. The active region on the column is indicated in Fig. 3*B*. It appears at approximately an R_F (relative to the marker dye) of 0.3 to 0.4. When the active fraction was again submitted to electrophoresis, bands of higher and lower R_F were absent (Fig. 2*B*). When, however, the active fraction 8 from a Bio-gel P-200 column was submitted, the resulting pattern of bands comprised a larger range of R_F values (Fig. 2*C*).

There is no reason to assume that bursicon is represented by any of the visible bands in the active region. The same bands appear in this region with fraction 4 from the Bio-gel column which is inactive and contains the large-sized proteins (Fig. 2*D*), and with fraction 8 which is active (Fig. 2*C*). When pooled samples of active fractions eluted from the gel were concentrated (Aquacide) and again submitted to electrophoresis, no difference was found between this band pattern and that of a control with corresponding fractions from blood with no activity. Thus either the position of bursicon in electrophoresis coincides with that of other bands which we have not been able to exclude, or, more likely, the quantity of active protein is so small that it cannot be detected by the usual staining methods.

Bursicon activity is located in the median neurosecretory cells (MNC) of the brain, and in the abdominal region of the thoracico-abdominal ganglion, as well as in the blood (*4*). Extracts from the ganglion are about six times more active than those from the brain (*4*), which raises questions as to the functional relationship of the bursicon activity found in these tissues and, indeed, the chemical identity of the underlying principles. To this purpose, extracts from the MNC, and from the abdominal region of the ganglion were submitted to the same treatments as had been applied to blood, namely, electrophoresis, gel filtration, precipitation with $(NH_4)_2SO_4$, heat treatment, and dialysis.

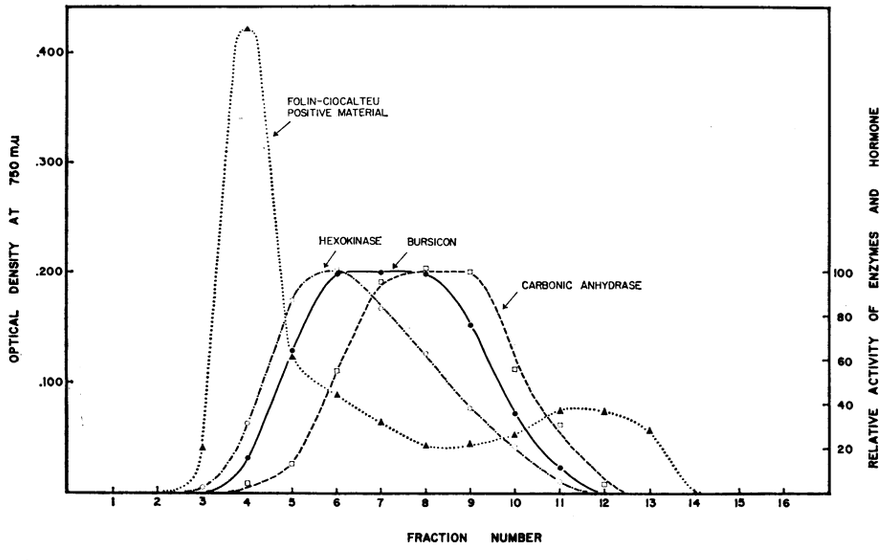

Fig. 1. Gel filtration (Bio-gel P-200) of a mixture of fly blood containing bursicon activity and the enzymes hexokinase and carbonic anhydrase. The large peak of Folin-Ciocalteu positive material in fractions 4 and 5 indicates blood proteins, and the small rise in fractions 11 and 12 is probably due to polypeptides and amino acids.

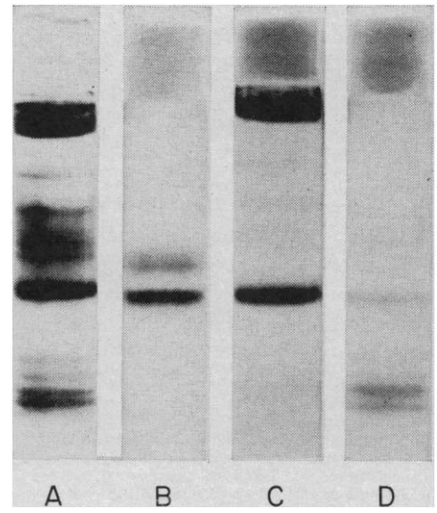

Fig. 2. Polyacrylamide disc electrophoresis pattern of: (*A*) active fly blood; (*B*) the active (No. 6) zone from *A*, subjected again to electrophoresis; (*C*) the inactive fraction No. 4 from Bio-gel P-200 filtration; (*D*) the active fraction No. 8 from Bio-gel P-200 filtration.

The MNC of 100 flies were dissected out, ground in Ringer, and the extractions passed through a column of Bio-gel P-200. Activity was located in fractions 7 to 9, that is, the same region as in corresponding experiments with blood containing activity. Exactly the same location of activity ensued when extracts from 100 abdominal regions of the thoracic ganglion were passed through the column.

The electrophoresis pattern of extracts of activity from the neurosecretory cells of the brain differed from that of blood. In the cells activity located in two widely separated regions, one identical with that of blood and the other representing the fastest moving fractions. In three different experiments the active fractions were 1–2 and 7–8, 1–3 and 7, and 1 and 8–9 (Table 1), respectively. On electrophoresis, extracts from the ganglion showed a single peak at fractions 6–7 (Table 1), which coincides with that from blood and one of the peaks from the neurosecretory cells.

A considerable portion of bursicon activity is destroyed on heating active blood for 3 to 5 minutes at 100°C, losses in activity varying between 40 and 90 percent in different experiments (*4*), but whether this loss was due to direct effect of temperature on bursicon or was caused by the adsorption of hormone on precipitating blood proteins was not determined. When ex-

tracts from neurosecretory cells of the brain were submitted to a similar heat treatment, little or no activity was lost. Since this difference might have been due to the much smaller amount of protein precipitating from brain-cell extracts than from blood, the experiment was repeated with blood samples which had been diluted ten times, and MNC extracts prepared in Ringer solution to which 10 percent inactive blood had been added. The same difference in temperature lability was observed. This would indicate that bursicon in the blood is much less heat stable than the hormone in the MNC. Extracts from the ganglion behaved the same way as neurosecretory cell extracts.

The hormone activity of the blood was nondialyzable and was precipitated by $(NH_4)_2SO_4$ without loss of activity (*4*). Extracts from the neurosecretory cells and the ganglion reacted essentially in the same way. These experiments, together with the results from gel-filtration and electrophoresis, dispose of the idea that the hormone in the brain might be chemically and functionally entirely different from bursicon and act merely as a trigger for the release of bursicon from the ganglion, in the same sense as a brain hormone releases ecdyson from the prothoracic glands. The hormone in brain and ganglion shows almost the same properties as that in the blood, the only noticeable difference being the greater lability to heating in the blood and the appearance of a second peak of activity on electrophoresis of extracts from brain cells. In our present stage of knowledge it would be futile to attempt an interpretation of these differences.

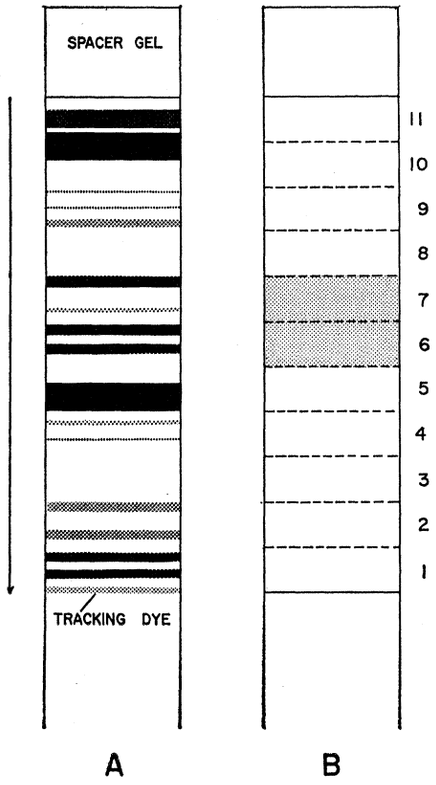

Fig. 3. (*A*) Interpretation of disc electrophoresis pattern of active blood (Fig. 2*A*). (*B*) Location of bursicon activity on the column after electrophoresis of active blood. The material moves in the direction of the arrow toward the anode.

Bursicon activity was also demonstrated in the blood of the roach *Periplaneta americana* at the time of emergence, and in the ganglia and corpora cardiaca at all times (*4*). Recently it was reported that the last abdominal ganglion of roaches plays a special role in tanning (*10*). These, and similar observations with other insects (*4*), have suggested that the tanning hormone of insects is unspecific. A comparison was made of active fractions from the blood, last abdominal ganglion, and corpora cardiaca from *P. americana* with corresponding fractions from flies. The relatively low hormone activity in roach blood has so far precluded the use of gel-filtration with its concomitant dilution of activity. Electrophoresis of roach blood, for reasons so far unexplained, did not lead to a clear-cut separation of hormonal activity. The active constituent from the last abdominal roach ganglion migrated in electrophoresis to the same locations 6–7 as with the various fly preparations (Table 1). Extracts from the corpora cardiaca showed exactly the same division of peaks of activity into two fractions (Table 1) as reported above for the neurosecretory cells of the fly. These results quite unexpectedly illuminate and support the fact that the corpora cardiaca constitute a storage organ for the brain hormone (*11*).

Evidence of protein nature has been claimed before, on the basis of reactions to precipitating agents, dialysis, and proteases, both for a brain hormone of the pupa of *Phil'osamia* (*12*) and for a diabetogenic hormone in the crustacean eyestalk (*13*).

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Chromosome Complement: A Fertile Hybrid between Equus prjewalskii and Equus caballus

Abstract. *At the Zoological Garden of Antwerp it has been proved that when* Equus prjewalskii *is crossed with* E. caballus *the offspring is fertile. As expected, its diploid chromosome number is 65. The morphological differences between the parental and the offspring karyotypes are not great; therefore, meiosis in the hybrid was successfully achieved.*

A difference between the diploid chromosome numbers of *Equus caballus* ($2N = 64$) and *E. prjewalskii* ($2N = 66$) has recently been reported (*1*). For a long time, one of us was of the opinion that *E. caballus* and *E. prjewalskii* in fact represented two different species of the horse family. As no living hybrids were known to exist, experimental breeding between Prjewalski's horse and the domestic mare was undertaken at the Zoological Garden of Antwerp. Two races of *E. caballus*, the Norwegian fjord pony and the Pyrenees pony, had been selected for this purpose since they were geographically the most distant from Mongolia, the habitat of *E. prjewalskii*. Further experiments proved that one of the two resulting male hybrids was fertile (*2*). This stallion, born in March 1962, impregnated late in 1964 a female Pyrenees pony, which gave