# Phosphatase Activity of *Drosophila* Salivary Glands

BY

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THE presence of alkaline phosphatase in chromosomes has been demonstrated by means of histochemical staining methods (Danielli, 1946; Krugelis, 1942, 1946). These studies indicated the effects of various fixatives and substrates on the subsequent staining reactions. The present study presents certain quantitative aspects of the effects of acetone, alcohol, and pH on the phosphatase activity of whole salivary glands of *Drosophila*.

## Material and Methods

Paranitrophenyl phosphate (Bessey, Lowry, and Brock, 1946) was used as a substrate. Magnesium was omitted after failure to detect increased activity with added magnesium. Hydrolysis was proportional to enzyme concentration under the conditions employed. In order to reduce the rate of inactivation of the enzyme the determinations of alkaline phosphatase activity were made at pH 8·6 rather than at the more alkaline optimum of maximal activity.

To determine the general characteristics of the phosphatases present in this material, several dozen glands were pooled and attempts made to get reproducible extracts and suspensions. This method was unsatisfactory. The salivary glands vary greatly in size and enzyme activity from one individual to another; but the single glands of a pair appear to be identical in size and enzyme content. Accordingly it is possible to control factors such as differential extraction of the enzymes and individual variation as follows: provided that enzyme blanks (the paranitrophenol colour value of samples without substrate) are negligible, as is the case here, each larva provides two aliquots. A pH curve or other comparison can then be run as indicated in Table I, where letters represent larvae and postscripts the glands of the pair.

Table I

| pH | 4·6 | 5·0 | 5·4 | 5·8 | &c. |
|---|---|---|---|---|---|
| Samples . . . . | $A_1$ | $A_2$ | | | |
| | | $B_1$ | $B_2$ | | |
| | | | $C_1$ | $C_2$ | |
| | | | | $D_1$ | &c. |
| | $K_1$ | | $K_2$ | | |
| | | $L_1$ | | $L_2$ | |

Individual glands were dissected in saline and fixed in acetone in tubes 6×25 mm. The acetone was replaced three times during the first 10 minutes; at the end of the fixation period all but 1·0 c.mm. of acetone was removed and 50 c.mm. of buffer added (M/25 diaminobutane pH 7·3–10·2, or barbital pH 3·0–7·3). After a suitable extraction period 50 c.mm. of substrate (adjusted to the pH of the extraction buffer) were added. The preparations were incubated 1–3 hours at 30° C., depending on size of gland. The gland was removed with a glass needle and the reaction stopped by addition of 15 c.mm. of 1·0 N. NaOH. The optical density of 100 c.mm. samples was measured on a Beckman spectrophotometer using Lowry micro-cells. Enzyme blanks were run on some pairs in each series but their colour was invariably negligible. The glands remained intact through several consecutive determinations.

## Observations

*Fresh extracts.* When fresh glands were placed in distilled water or buffer for varying periods prior to addition of the substrate very little enzyme activity was found. In one case 20 glands were placed in 100 c.mm. of distilled water and subjected to the gentle pounding of an electromagnetic stirring bead for 2 hours. In this instance less than 10 per cent. of the enzymatic activity of the acetone-fixed aliquot was found. In preliminary experiments it was found that whenever, during dissection, a cell of a gland was penetrated by the dissecting needle, the subsequently determined activity of the gland was higher than that of the uninjured gland (cf. Table IV, B3 and B4). It is evident that either the cell membrane or more probably the membranous capsule of the gland effectively prevents extraction of the enzyme and entrance of the substrate even in unphysiological media.

This fairly obvious property of membranes has occasionally been measured (Linderstrøm-Lang and Holter, 1933, and Doyle, 1938). When the gland is frozen (Table II, B1, B2; C1, C2) or is fixed (Table II, Q1, Q2; R1, R2) this property of the gland surface is diminished.

Table II. *Phosphatase Activities of Individual Salivary Glands*

| *Gland* | *Treatment* | *pH* | *Activity* |
|---|---|---|---|
| B1 | Fresh | 8·6 | 0·90 |
| B2 | Frozen | 8·6 | 1·37 |
| C1 | Fresh | 8·6 | 0·160 |
| C2 | Frozen | 8·6 | 0·400 |
| Q1 | Fresh | 8·6 | 0·047 |
| Q2 | Acetone 20 min. | 8·6 | 0·321 |
| R1 | Fresh | 8·6 | 0·056 |
| R2 | Acetone 24 hrs. | 8·6 | 0·400 |
| S1 | Acetone 24 hrs. | 9·5 | 0·700 |
| S2 | Acetone 24 hrs. | 8·6 | 0·472 |
| Fat | Acetone 24 hrs. | 8·6 | 0·035 |

Fat is found closely applied to the glands and it is sometimes difficult to remove all of the fat without injuring the glands. That small traces of fat contain negligible quantities of phosphatase is shown in Table II, where pieces of fat twice the volume of the glands of specimen S were assayed along with the glands.

*Fixed glands.* Several pairs of glands were fixed in acetone for 24 hours and allowed to stand in distilled water for 15 minutes. Negligible activity was found in the extract and the activity of the extracted gland corresponded with the activity in glands not extracted. When extracted for 24 hours at 5° C. in M/25 diaminobutane buffer pH 9·5, about one-third of the total activity was found in the extract. In another series glands fixed in acetone for 9 days and extracted for 16 hours at 5° C. with M/50 barbital pH 7·3 gave 20 per cent. of the total activity in the extract. In this series redetermination of the residual phosphatase activity gave 87 per cent. of the initial residual activity. The alternate glands of pairs treated at the same pH with diaminobutane buffer gave corresponding results.

The total enzymatic activity of glands fixed in acetone is higher (at pH 4·4 and 8·6) than that found after similar treatment with 80 per cent. alcohol (Table III). Here 75 per cent. of the pH 8·6 acetone value was found after

TABLE III. *Phosphatase Activities of Glands after Fixation in 80 per cent. Alcohol and in Acetone at pH 8·6*

| Gland | Time of fixation | | Observed activity | pH | Notes |
|---|---|---|---|---|---|
| | *Alcohol* | *Acetone* | | | |
| H1 | 2 hrs. | .. | 0·408 | 8·6 | = 70 per cent. of H2 (Alk-p-ase). |
| H2 | .. | 2 hrs. | 0·590 | 8·6 | |
| J1 | 1½ ,, | .. | 0·175 | 8·6 | = 75 per cent. of J2. |
| J2 | .. | 1½ ,, | 0·235 | 8·6 | |
| K1 | 2½ ,, | 22 ,, | 0·185 | 8·6 | = 77 per cent. of K2. |
| K2 | 0 | 24 ,, | 0·248 | 8·6 | |
| L3 | 2½ ,, | 22 ,, | 0·119 | 8·6 | = 73 per cent. of L4. |
| L4 | 0 | 24 ,, | 0·171 | 8·6 | |
| M7 | 2½ ,, | 22 ,, | 0·110 | 4·4 | = 20 per cent. of M8 (Acid p-ase). |
| M8 | 0 | 24 ,, | 0·585 | 4·4 | |

80 per cent. alcohol treatment and 20 per cent. of the pH 4·4 acetone value. Since the results obtained with specimens J and H might be ascribed to a differential effect of acetone and alcohol on the penetrability of the cellular material, the subsequent specimens (K, L, M) were treated with acetone as well after the alcohol fixation.

*Redeterminations on the same gland.* Using whole acetone-fixed glands, only a fraction of the enzyme is extracted during the initial determination. A second determination may then be made under similar or altered conditions to examine the effect of the first determination on the residual enzyme.

Representative data from these experiments are given in Table IV. Here it would appear that 60 per cent. of the pH 8·6 activity survives a determination (Detn.) at pH 4·4; that 45 per cent. of the pH 4·4 activity survives a determination at pH 8·6; that 38 per cent. of the pH 8·6 activity survives a determination of pH 8·6; and that 87 per cent. of the pH 7·3 activity survives a determination at pH 7·3. The decreased activity after the more alkaline initial determinations is in keeping with other findings (Lundsteen and Vermehren, 1936) in which the pH optimum is more alkaline for short digestion times than for longer ones.

TABLE IV. *Redetermination of Phosphatase Activities on Glands fixed in Acetone for 24 Hours*

| | *pH* | | *Activity* | | |
|---|---|---|---|---|---|
| *Gland* | *Detn. 1* | *Detn. 2* | *Detn. 1* | *Detn. 2* | *Notes* |
| T1 | 7·3 | 7·3 | 0·990 | 0·870 | $\frac{870}{990}$ = 87 per cent. of p-ase after neutral Detn. |
| A1 | 4·4 | 8·6 | 0·317 | 0·244 | $\frac{244}{413}$ = 60 per cent. of Alk-p-ase after acid Detn. |
| A2 | 8·6 | 4·4 | 0·413 | 0·150 | $\frac{150}{317}$ = 45 per cent. of Ac-p-ase after alk. Detn. |
| B3 | 8·6 | 8·6 | 0·351 | 0·133 | B4 punctured with needle. $\frac{133}{351}$ and $\frac{183}{462}$. |
| B4 | 8·6 | 8·6 | 0·462 | 0·183 | = 38–9 per cent. of Alk-p-ase after alk. Detn. |

Gomori preparations for both acid and alkaline phosphatase made subsequently to the initial quantitative determinations showed little differences from those made immediately following the respective fixations.

## DISCUSSION

That phosphoesterases from different sources vary with respect to stability to acetone and to alcohol is indicated by comparison of these results with those of Stafford and Atkinson (1948, rat tissues), who found higher alkaline phosphatase activity after alcohol (80 per cent.) than after acetone fixation.

The absence of appropriate values for fresh extracts and homogenates in this study prevents an estimation of the degree of destruction of alkaline and acid phosphatase by the alcohol and acetone. Determination of the ratio of activities found to true enzyme content requires the preparation of suitably cytolysed samples without major losses.

It is obvious, however, that acetone preserves more phosphatase activity in our material than does alcohol.

In specimen B, Table IV, B4 was deliberately punctured during dissection with resulting higher activity. The amount of enzyme lost between deter-

mination 1 and 2 was essentially the same in glands B4 and B3 (unpunctured). This would indicate that the increased activity of B4 over B3 is due to better access of substrate, and that at pH 8·6 (for 2 hours) the decreased activity in determination 2 as compared to the initial determination is due to inactivation rather than to extraction of the enzyme.

With sectioned rabbit material (Doyle—in the press) there is much greater extraction of enzyme (especially of acid phosphatase) than from these whole glands. Furthermore, acid phosphatases from many sources are much more labile than alkaline phosphatases. Gomori (1946) used acetyl cellulose to reduce losses and translocation of enzyme. A coating on the section which prevents diffusion of the enzyme will to some degree also affect the rate of penetration of the substrate. The problem then arises as to whether, in the absence of completely cytolysed cells in extracts and in the absence of free access of substrate to cell structure in sections, the activities observed under given experimental conditions can represent the enzymatic contents of the material. Only when the true enzymatic contents of cellular structures can be ascertained will there be a better basis for conjecture concerning the role of the enzymes, but the demonstration of maximal enzymatic *content* will not of itself indicate the normal physiological *activity* with respect to that enzyme.

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## Summary and Conclusions

The phosphatases in the cytoplasm and nuclei of *Drosophila* salivary glands are better preserved by fixation in absolute acetone than in 85 per cent. alcohol. In whole glands there is relatively little extraction of the enzyme during assay. Phosphatase activity is more resistant to incubation at neutrality than at pH 8·6, but in this material there is sufficient residual enzymatic activity to permit redetermination of alkaline, neutral, or acid phosphatase activity by staining methods after an initial quantitative determination. The state of the membranes of the gland affects the penetration of the substrate sufficiently to limit the activities obtained.

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