# EPINEPHRIN-ACTIVATED AMYLASE SECRETION IN PAROTID SLICES AND LEAKAGE OF THE ENZYME IN THE COLD[1]

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It has been previously reported that slices of rat parotid gland actively secrete $\alpha$-amylase when epinephrin is added to the incubation medium (Bdolah et al., 1964). Release of enzyme in the absence of epinephrin appeared to be due solely to passive leakage. The rate of leakage was, however, quite high and the effect of the hormone was limited to a two to four fold increase in the rate of enzyme release.

Recent experiments have shown that amylase leakage from the slice during incubation at 37° is due largely to the repeated exposure of the glands and slices to cold medium during preparation and after preincubation. It has also been found that zymogen granules isolated from the homogenate of the gland in 0.3 M sucrose, become leaky in the cold and release the amylase accumulated inside. The findings indicate an effect of temperature on the lipid structure of the zymogen granule membrane. Some pertinent experiments are described in this communication.

Kinetics of enzyme secretion measured on slices which had not been exposed to cold medium during preparation, are given in Fig. 1. It is evident

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Abbreviations: KRB, Krebs Ringer bicarbonate medium; BSA, Bovine serum albumin.

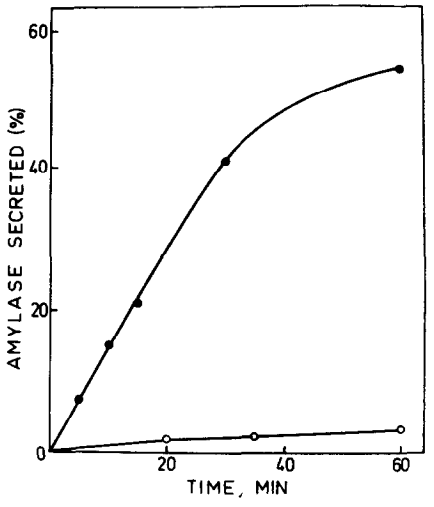


Fig. 1. Amylase secretion by rat parotid slices prepared at 37°.

The experimental system, preincubation and other methods were as previously described (Bdolah et al., 1964), but for the following important modification. Glands were collected and slices were prepared in KRB medium at 37° which was continuously gassed with a mixture of 95% $O_2$, 5% $CO_2$. In contrast to the previous procedure, glands were not exposed to cold medium during any stage of the experiment. Each of the two vessels contained slices equivalent to one gland (about 6,000 amylase units). ●———●, 0.01 mM epinephrin; O———O, control without epinephrin.

Definition of percent secretion: The amount of amylase released into the medium plus the amount finally remaining in the slice (measured in the homogenate) is defined as 100%. The amount secreted during any time interval is thus expressed as percent of total.

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that these slices show very little amylase leakage so that release of enzyme into the medium is almost entirely dependent on the addition of epinephrin. The low rate of enzyme leakage permitted assessment of the time period required for activation of the secretion process by epinephrin. Fig. 2 shows that enzyme secretion is already in progress at a linear rate 30 seconds after addition of epinephrin. It may therefore be estimated that penetration of the hormone into the cells, activation of amylase release from the intracellular zymogen granules (Bdolah et al., 1964) and transport of the enzyme from the cells into the medium, require altogether less than ten seconds.

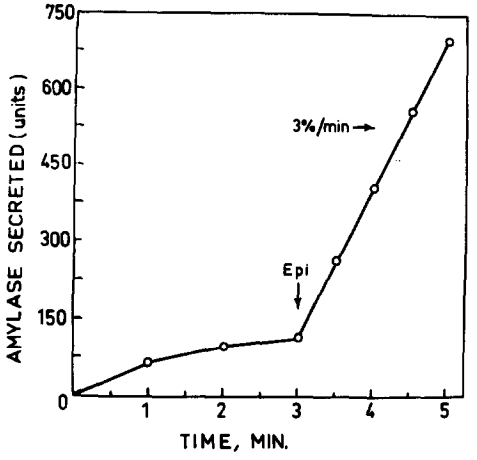


Fig. 2. Onset of amylase secretion by addition of epinephrin to the medium.

Slices equivalent to 3 glands containing 10,000 amylase units were prepared at 37° (see under Fig. 1), preincubated and transferred into 5 ml fresh KRB medium. At points indicated on the curve, 0.05 ml aliquots of the medium were removed for assay of amylase released into the medium. Epinephrin, 0.01 mM was added at point marked with arrow.

The rate of enzyme secretion after addition of the hormone corresponds to 3% of the total amylase in the slice released per minute (indicated by arrow).

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The finding that incubation in the cold causes leakage of amylase from the gland cell suggested the possibility of a direct effect of low temperature on the zymogen granules which contain most of the intracellular amylase. As shown in Table 1, zymogen granules in isotonic sucrose or in KCl solution lose a large portion of their amylase content when incubated at 0° or even at 15°. The small amount of enzyme released at 37° probably represents damage of the granules during preparation since this value does not increase appreciably even when incubation is prolonged to 60 minutes. It should be noted that the granules may be isolated in the cold with only slight losses in amylase content since cold lability becomes pronounced only after isolation from the homogenate and washing of the granule fraction. The washed

fraction prepared in the cold is, however, unstable unless it is transferred to 37° (Table 1).

Table 1

Cold lability of zymogen granules

| Temp. at which granules were prepared | Test Medium | Amylase released from the granules, % of total | | |
|---|---|---|---|---|
| | | 0° | 15° | 37° |
| 37° | Sucrose | 75 | 25 | 10 |
| 0° | Sucrose | 25 | - | 10 |
| 37° | KCl | 100 | - | 16 |

37° preparation: Four glands, one each from four starved rats, were collected in KRB medium at 37° as outlined under Fig. 1. A homogenate was prepared at 30° in 0.3 M sucrose containing 0.01 M Tris buffer pH 7.5 and 0.5 mM EDTA. The crude zymogen granule fraction was isolated by centrifugation at room temp. for 10 min. at 1,200 x g. The fraction was washed once, and finally suspended at 30° in 2 ml of the above sucrose medium.

0° preparation: The other four glands, from the same rats which served for the 37° preparation, were collected directly in ice cold sucrose, Tris, EDTA medium. The crude zymogen granule fraction was isolated by the same procedure described above but all operations were carried out at 0°.

Test of amylase release: 0.1 ml aliquots of the zymogen granule preparations, containing about 500 amylase units, were added to 0.9 ml of test medium. The test medium contained 10 mM Tris buffer pH 7.5, 0.5 mM EDTA with either 0.3 M sucrose or 0.15 M KCl. The granule suspensions were incubated for 15 min. at the temperatures indicated in the Table and centrifuged at room temp. for 10 min. at 2,000 x g. Enzyme released from the granules into the supernatant and total enzyme were measured.

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It seems most likely that leakage of amylase from the zymogen granule in the cold is due to a temperature effect on the granule membrane. The membrane contains phospholipid and possibly other lipid components (Schramm and Danon, 1961). Studies of films of fatty acids and other lipids have long established that their surface area decreases abruptly with decreasing temperature within a temperature range which is characteristic for each lipid (Adam, 1941). It might therefore be expected that

lipid components in the zymogen granule membrane would 'contract' in the cold to open gaps which permit leakage of amylase. This hypothesis suggests that addition of the proper lipid would protect against leakage in the cold by replenishing the deficient areas in the membrane. It is shown in Table 2 that long chain fatty acids, when tested at concentrations sufficiently low not to cause lysis, indeed protect against leakage of amylase in the cold. This effect appears to be fairly specific for long chain fatty

Table 2

The effects of long chain fatty acids and serum albumin on amylase release from the zymogen granules

| Incubation Temperature | Additions | mM | Amylase released from granules, % of total |
|---|---|---|---|
| 37° | None | | 10 |
| 0° | None | | 30 |
| 0° | Laurate | 0.05 | 14 |
| 0° | | 0.25 | 8 |
| 0° | | 1.00 | 42 |
| 0° | Oleate | 0.02 | 16 |
| 0° | | 0.10 | 85 |
| 0° | Palmitate | 0.04 | 15 |
| 0° | Fatty acid 'free' BSA, 0.2% | | 62 |
| 0° | Fatty acid 'free' BSA, re-saturated with palmitate, 0.2% | | 25 |
| 37° | Fatty acid 'free' BSA, 1% | | 38 |

Zymogen granules were isolated and release of amylase was measured as described under Table 1 for the 37° preparation. However, 0.01 M phosphate buffer pH 7.5 replaced Tris buffer in the sucrose EDTA medium in all stages of the experiment. Fatty acids and BSA preparations were added to sucrose test medium at 37° prior to the addition of zymogen granules.

Fatty acid 'free' BSA was prepared by repeated extraction of crystalline BSA at 0° with absolute ethanol and finally with ether. The preparation was then dialyzed overnight against 0.01 M Tris buffer pH 7.5 and finally against distilled water. To resaturate the BSA with palmitate, five mole equivalents of the fatty acid were added.

acids since octanoate, cholesterol and a sonicated mixture of phospholipids (Conover et al., 1963) did not prevent leakage in the cold.

BSA which effectively binds free fatty acids was shown to increase amylase leakage from the granule both at 0° and at 37° while BSA saturated with palmitate was inert (Table 2). These findings suggest that fatty acids may represent the essential, cold sensitive, component of the membrane.

The question of cold lability of cellular and subcellular structures has often been ignored due to the widespread custom of handling all such preparations in the cold. Experiments on bacterial cultures show that brief cooling may cause release of intracellular enzymes (Pollock, 1961) and a precipitous drop in viability (Meynell, 1958). In both of these reports the cell membrane is implicated as the cold labile structure affected.

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