

# THE PACKAGING OF A SECRETORY PROTEIN

## Kinetics of Cocoonase Zymogen Transport into a Storage Vacuole

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### INTRODUCTION

The transport of secretory proteins from membrane-bound polysomes to extracellular space is a key activity in a variety of cells; nevertheless, it is still incompletely understood. Much of the available information has come from a limited number of mammalian tissues, especially pancreas (1, 2, 3, 4).

The zymogen cells of silk moth galea are unusually favorable for studies of secretory protein transport. These cells are highly specialized for large-scale secretion of a single protein, the zymogen of cocoonase (5). After its synthesis, zymogen is quantitatively sequestered into a single, large storage vacuole in a pure form (6), facilitating the monitoring of transport by radioautography (7). The cells can be maintained for at least 2 days in organ culture at a variety of developmental stages. In vivo, during a 7 day differentiated phase (8), zymogen synthesis progressively accelerates, while synthesis of nonsecretory proteins decreases; moreover, by the use of actinomycin D, synthesis of proteins other than zymogen may be virtually abolished (7). Finally, these invertebrate cells afford a unique opportunity for comparative studies of secretion. The present report concerns the kinetics of zymogen transport into the storage vacuole of cultured galea cells at various developmental stages.

### MATERIALS AND METHODS

Galeae were dissected and cultured in serum-free Grace's (9) medium (Grand Island Biological Co., Berkeley, Calif.) as previously described (7). Each galea was cut transversely into three segments; thus, six segments were available per animal. All tissues were given a simultaneous 15 min pulse of L-leucine-4,5-<sup>3</sup>H (50  $\mu$ Ci/ml, 0.57  $\mu$ moles/ml), washed three times with nonradioactive medium (containing 0.57  $\mu$ moles/ml L-leucine-<sup>1</sup>H), and "chased" for various periods of time; thereafter, they were fixed in 6% glutaraldehyde containing 40% Weevers' (10) pupal saline, dehydrated in alcohols, and embedded in glycol methacrylate (11); no soluble radioactivity re-

mained in the tissue after such processing. Sections were cut at 1  $\mu$  thickness, coated with NTB-2 Kodak liquid emulsion, exposed for 10 or 20 days at 4°C, developed, and stained with 0.05% toluidine blue. Grain densities were determined as follows. Representative sections were photographed and printed at a final magnification of 1350 or 1750. On the prints, areas representing the general cytoplasm, nucleus, and vacuole were outlined, measured by planimetry, and the grains within each counted. The results were expressed as grain densities (average number of grains per unit area at a standard magnification), and are based on 100–3200 grain counts (100–3200 for vacuole, 400–2100 for cytoplasm, 100–300 for nucleus). Data were also corrected for background and standardized for radioautographic exposure. The

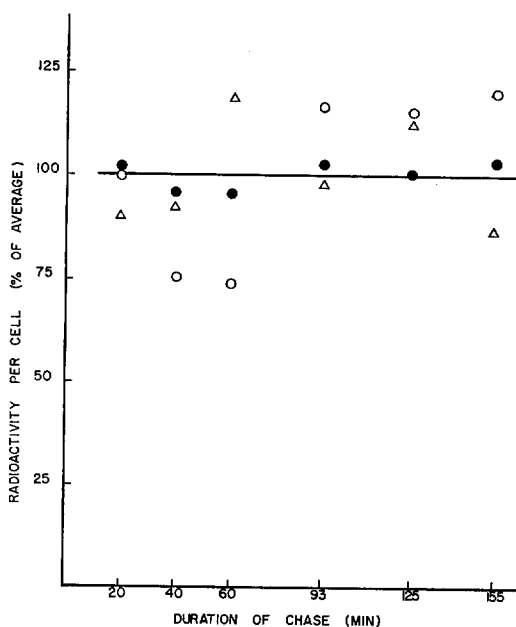


FIGURE 1 Total radioactivity incorporated per cell as a function of duration of chase. Values are expressed as per cent of the average incorporation for the six chase periods of the same experiment.  $\Delta$ , experiment using a day 10 animal;  $\bullet$ , day 12;  $\circ$ , day 15. Constancy of label indicates an effective pulse-chase regimen.

total areas of vacuole, cytoplasm, and nucleus per cell were determined, and thus grain densities could be translated into total grains per cell compartment.

## RESULTS

Experiments were performed on the galeae of three *Antheraea polyphemus* at different stages of adult development (5): days 10 (less than 24 hr after initi-

ation of cocoonase synthesis), 12, and 15 (less than 24 hr before cessation of synthesis). Six segments from each animal were labeled with leucine- $^3\text{H}$  for 15 min and subsequently cultured in cold medium for chase periods ranging from 20 to 155 min.

Fig. 1 shows the total number of grains per zymogen cell as a function of chase period. No significant consistent change in radioactivity per cell

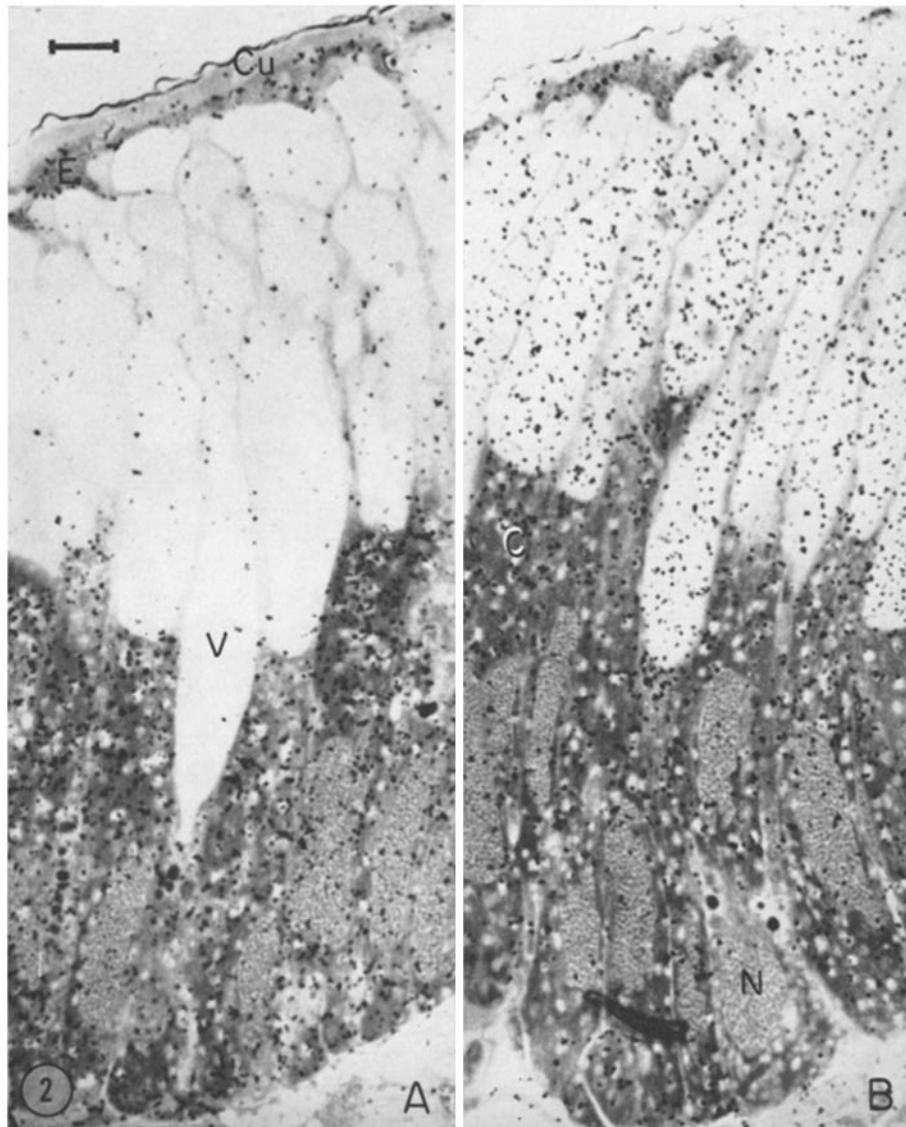


FIGURE 2 Transfer of radioactivity from cytoplasm to vacuole in zymogen cells. A, 20 min chase; B, 93 min chase. *N*, nucleus; *C*, cytoplasm; *V*, vacuole; *Cu*, cuticle; *E*, epidermal cell. The external surface of the gland is uppermost, the blood below. Day 15 galea, 20 day radioautographic exposure. Magnification, 930 (scale, 10  $\mu$ ).

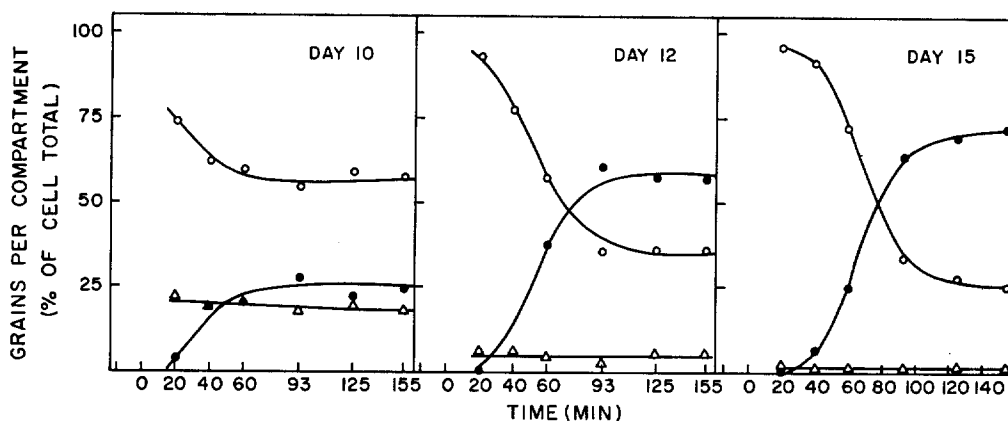


FIGURE 3 The effect of chase duration on subcellular localization of label in zymogen cells at three developmental stages. The label in each compartment is shown as per cent of total cell label. Since the total remains approximately constant (Fig. 1), the curves also indicate absolute acquisition or loss of label from each compartment. The first mark on the abscissa indicates the beginning of the pulse, and time zero is the beginning of the chase period.  $\circ$ , cytoplasm;  $\bullet$ , vacuole;  $\Delta$ , nucleus.

was noted during chase. For such brief periods, protein turnover or release of labeled protein into the culture medium are inconsequential (unpublished observations). Thus, Fig. 1 indicates efficient chase after a true pulse. This permits us to normalize results in terms of total grains per cell, so as to minimize errors introduced by random variations in incorporation; normalized results still reflect absolute changes in radioactivity.

Movement of labeled protein from cytoplasm to vacuole is documented in Fig. 2. After 20 min, virtually no grains are found in the vacuole (Fig. 2 A). After 93 min, appreciable radioactivity has reached the vacuole, while cytoplasmic radioactivity has been reduced (Fig. 2 B). By contrast, nuclear labeling seems constant.

The quantitative distribution of label between cytoplasm, nucleus, and vacuole is plotted in Fig. 3 as a function of chase period. Despite differences in detail, the main features are similar in all three animals. After 20 min, radioactivity in the vacuole progressively increases from virtually zero until a plateau at about 60–120 min; cytoplasmic radioactivity shows a corresponding decrease, while nuclear label is nearly stable. The “plateau” distribution is maintained, even after a day (7). Thus, labeled zymogen is completely transferred into the vacuole within the time of the present experiments.

The kinetics of transport become clear with the realization that labeled zymogen is exclusively and totally represented by the label which migrates from cytoplasm to vacuole. This migration can be

treated as a precursor-product conversion: “cytoplasmic” zymogen is converted to “vacuolar” zymogen. Differences in radioactivity between time zero and final plateau levels can be interpreted, in the case of cytoplasm, as a decrease of cytoplasmic zymogen from 100% to 0% and, in the case of vacuole, as an increase of vacuolar zymogen from 0% to 100%. For any particular animal, the curves of zymogen disappearance from cytoplasm and appearance in vacuole should be mirror images, intersecting at the 50% point and thus defining the half-transport time,  $t_{50}$  (the time, measured from the middle of the pulse-labeling period, necessary for half the zymogen to be transported). The actual curves (Fig. 4) indicate that  $t_{50}$  is progressively greater in cells from older animals: 38 min on day 10, 60 on day 12, and 74 on day 15.<sup>1</sup>

The plateau levels of radioactivity in Fig. 3 and the absolute grain counts confirm that during the

<sup>1</sup> The main errors in these estimates are two. First, although all vacuolar grains are assigned to zymogen (see legend of Fig. 4), phase contrast reveals that on day 10 some, in fact, represent labeling of an intravacuolar duct (5); however, duct label is a minor fraction, even at the shortest chase period. Second, for Fig. 4 we assumed that nuclear label is stable, although it may decrease imperceptibly (Fig. 3); however, such a small decrease is probably attributable to progressively smaller contributions of perinuclear cytoplasm. Thus, the values of  $t_{50}$  do not change significantly if corrected for these errors.

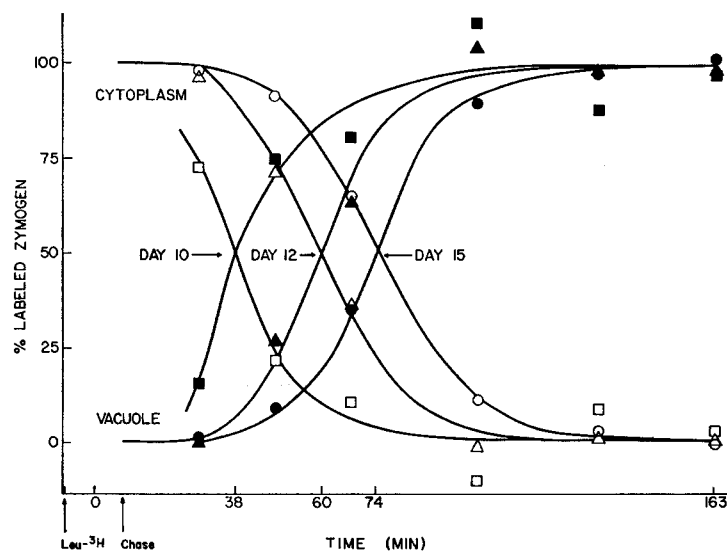


FIGURE 4 Kinetics of zymogen transport from cytoplasm to vacuole, based on Fig. 3. The middle of the pulse is set as time zero. Changes in total radioactivity of cytoplasm or vacuole reflect the transport of zymogen; increases or decreases (Fig. 3), between time zero and any particular time point, are expressed as per cent of the over-all change from time zero to the plateau level in the particular cell compartment. The time zero radioactivity of the vacuole is estimated as zero, and of the cytoplasm as 100% minus the highest nuclear radioactivity. The intercepts at 50% define the half-transport time,  $t_{50}$ . □, day 10 cytoplasm; ■, day 10 vacuole; △, day 12 cytoplasm; ▲, day 12 vacuole; ○, day 15 cytoplasm; ●, day 15 vacuole.

differentiated phase (days 9–16) synthesis of cytoplasmic (i.e. nonexportable) and nuclear proteins progressively declines, while zymogen synthesis greatly increases, both in absolute and in relative terms (from 24% of the total protein synthesis on day 10 to 74% on day 15; also see reference 8).

#### DISCUSSION

When transport is viewed as a precursor-product conversion (Fig. 4), it becomes apparent that two main parameters are important for adequate description of transport kinetics: the shape of the transport curve, and its position relative to the time axis.

The half transport time,  $t_{50}$ , defines the position on the time axis. In terms of physical meaning,  $t_{50}$  (the time required for an average zymogen molecule to move from the site of synthesis to the site of storage) clearly reflects the average duration of all transport steps.

The shape of the transport curve is approximately sigmoidal; it begins with a lag phase, during which little radioactivity moves, and then changes into a phase of rapid, nearly linear transport. Our results to date are obviously inadequate

for precise measurements of lag, but they do permit us to determine transport rates during the linear phase. Moreover, since  $t_{50}$  is influenced by both lag and linear phases, an approximate estimate of lag can be obtained indirectly. The lag represents the minimum time required for “processing” newly synthesized zymogen before it can enter the storage vacuole. Since the apical cytoplasm, nearest the vacuole, appears similar to other regions of the cell, containing endoplasmic reticulum and Golgi complexes (unpublished observations), it seems reasonable to assume that the lag represents the minimum “processing time” of molecules synthesized in the vicinity of the vacuole, and thus reflects the duration of all transport steps except long distance translocation within the cell. On the other hand, the per cent rate of label movement<sup>2</sup> during the linear phase is probably affected by three factors: the duration of pulse labeling, the inherent variability of all transport steps, and the time re-

<sup>2</sup> The absolute rate of label movement, which cannot be calculated from the data on hand, should not be affected by the translocation rate.

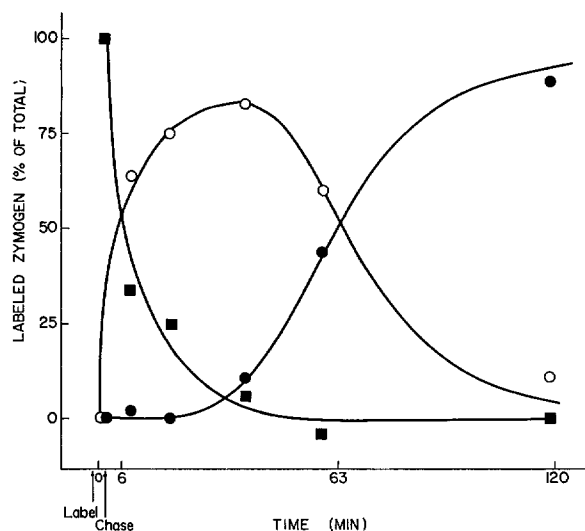


FIGURE 5 Kinetics of zymogen transport in guinea pig pancreas, based on the radioautographic data of Jamieson and Palade (3), with assumptions analogous to those used for Fig. 4. Transported label, i.e. zymogen, is 66–72% of the total radioactivity; the graphs represent per cent labeled zymogen found in each cell compartment at any particular time. ■, endoplasmic reticulum; ○, peripheral Golgi region and condensing vacuoles; ●, zymogen granules and acinar lumen.

quired for long-distance translocation (assuming that translocation has a significant duration).

The half transport time,  $t_{50}$ , substantially increases with age (Fig. 4). Since older cells are also longer (the long axis of the cytoplasm increases from *circa* 45  $\mu$  on day 10 to *circa* 80  $\mu$  on day 15; reference 5), one might ascribe this increase in  $t_{50}$  to the increased translocation distance. If so, one would predict that the lag phase remains constant and the linear per cent transport rate declines with age. This prediction is only partially correct. The per cent rate of transport,  $R$  (measured in the interval 35–65%) in fact declines, but only by one-quarter, from 2.7% on day 10 to 2.1%/min on day 15; by contrast,  $t_{50}$  nearly doubles. This indicates that the lag increases substantially with age (Fig. 4) and suggests that “local” zymogen processing is slower in older cells.<sup>3</sup>

The kinetics of zymogen transport in mam-

<sup>3</sup> Slower processing might result from the increased zymogen synthesis or from a decreased synthesis and consequent scarcity of key transport factors (e.g., membrane proteins, “processing” enzymes, ATP, etc). It should also be noted that the values of  $R$  and the cellular dimensions permit a rough estimate of translocation rate, 3.5  $\mu$ /min. Making allowance for true pulse duration, the variability factor inherent in  $R$  is 10 min for 100% transport.

malian pancreas and in insect galeae are surprisingly similar. Jamieson and Palade (3) have studied zymogen transport in guinea pig pancreatic slices; we have analyzed their electron microscopic radioautographic data quantitatively, in terms of the precursor-product model (Fig. 5). As the original authors point out, in the pancreas, transport from the endoplasmic reticulum to the periphery of the Golgi regions is very rapid:  $t_{50}$  for this step proves to be approximately 6 min. In the galea, although it is impossible to quantify intracytoplasmic movement merely with light-microscopic radioautography, it is noteworthy that even after the shortest chase grains are located primarily at the periphery of the unstained Golgi regions, rather than in basophilic areas corresponding to endoplasmic reticulum (Fig. 2 A). More significantly, the quantitative parameters of over-all transport to the site of storage are also similar. In the pancreas,  $t_{50}$  (for transport into zymogen granules and acinar lumen) is 63 min; in the galeae it varies from 38 to 74 min. In the pancreas,  $R$  is approximately 1.7%/min; in the galea, it is somewhat higher, 2.1–2.7%/min (even though the pulse was five times longer than in the pancreas). Thus, the galeal cells at 25°C function at least as efficiently as the pancreatic acinar cells at 37°C.

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