growth hormone in duplicate walks while ingesting glucose; he also showed similarly increased concentrations of fatty acids and growth hormone during a walk after fat had been introduced into the third part of the duodenum by nasogastric tube, as during a walk when starving. This finding agrees with the current concept that ingested fat is utilized only after its uptake by adipose tissue and subsequent release as fatty acids.

Three subjects walked 30, 37, and 45 km, respectively, at 6.4 km/hr while fasting; Fig. 3 shows the data for one of them. There was a slow decrease in concentration of blood sugar, and a more marked and steady increase in nonesterified fatty acids in plasma throughout the walk; data on respiratory quotient show progressive increase in the proportion of fuel derived from fat, which reached nearly 100 percent toward the end of the walk. If, as seems probable, the increase in nonesterified fatty acids resulted principally from the secretion of growth hormone, it is perhaps surprising that the hormone concentrations indicate a series of intermittent bursts of secretion rather than a steady increase with time. The same general pattern was found in the other two long walks. We suggest that growth hormone mobilizes fat by a series of "triggering" actions. Rabinowitz, Klassen, and Zierler (*9*) showed that nonesterified fatty acids in plasma were still increasing 35 minutes after a close arterial infusion of growth hormone was terminated.

Walks for ½, 1, 2, and 5 hours at 6.4 km/hr were performed by one subject, a 37-year-old male, in the fasting state. The recovery period was followed in detail for the two shortest walks. Concentration of growth hormone was less than 1 ng/ml immediately after the ½-hour walk, but rose to 5.7 ng/ml 45 minutes later and fell to less than 1 ng/ml during the next hour, showing that the concentration may be high even some time after a comparatively short period of moderate exercise. In the 1-, 2-, and 5-hour walks, peak concentrations of 22, 24, and 18 ng/ml were recorded after 1 hour. After the 1-hour walk the concentration fell exponentially to less than 1 ng, with a half-time of 22½ minutes, a rate of decrease identical with that which follows a single intravenous injection of the hormone (*10*). This suggests that secretion stopped at the end of this walk. In the two longest walks the rate of decrease was slower, suggesting that, if exercise is continued during the 2nd hour, secretion is reduced but not abolished. However, the pattern of concentration of growth hormone during the 2nd hour was little different whether the walk continued or not, whereas the concentration of nonesterified fatty acids always increased until the end of the walk and fell immediately thereafter. We suggest that some other factor must antagonize the nonesterified fatty acid releasing action of the growth hormone present during the recovery phase. It seems probable that secretion of insulin may increase at this time; the period is characterized by increase in ketone bodies, which have been shown to increase secretion of insulin (*11*).

Most of our experiments have been with men, but two women showed similar changes during a 12.8-km walk. Our subjects ranged in age from 19 to 54, but no age difference in response by growth hormone appeared.

These studies show that, during muscular exercise by normal human adults, unless exogenous carbohydrate is made available, the needs for fuel are increasingly met by mobilization of depot fat, and that secretion of growth hormone appears largely responsible for initiating and maintaining this process. The magnitude of the effect of exercise on concentrations of growth hormone is so great that precise control of energy expenditure is a prerequisite for any studies of the concentrations of this hormone in plasma.

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16 August 1965

Lysosomal and Free Acid Phosphatase in Salivary Glands of Chironomus tentans

Abstract. *In cells of salivary glands of last-instar larvae of* Chironomus tentans, *acid phosphatase activity is bound to (probable) lysosomes and a few other cell organelles. At the end of the pupal molt the salivary gland breaks down. While acid phosphatase in areas of nondegenerated cells is still restricted to the structures mentioned, in degenerated areas the enzyme is freely distributed in the cytoplasm.*

In vertebrates, many hydrolytic enzymes such as acid phosphatase are localized in cytoplasmic organelles termed lysosomes (*1*). It is assumed that in the process of cell breakdown the enzymes are released from the lysosomes and distributed throughout the cell. Biochemical and electron-microscopic data have been interpreted to support this idea (*1, 2*). This interpretation has been questioned, however, as possibly being based on artifacts resulting from the experimental procedures (*3*). In insects many tissues break down during the developmental transition from the larval to the adult stage in the course of metamorphosis. Lysosomes have never been convincingly shown to occur in insects, although their existence has been reported (*4*). In our studies of the relations between changes in gene-activity (puffing) patterns and enzyme patterns related to the breakdown of salivary-gland cells in *Chironomus tentans* (*5*), we not only found organelles resembling vertebrate lysosomes, but obtained clear evidence of the existence of free acid

phosphatase in definite developmental stages.

Our procedure was a modification of that used by Holt and Hicks (*6*) to demonstrate acid phosphatase by electron microscopy. Salivary glands, fixed in 6.25 percent glutaraldehyde in 0.1*M* phosphate buffer (*p*H 7.2) (*7*), were incubated in the Gomori medium for 3 hours at 18°C; after treatment with OsO_4 they were then embedded in Durcupan ACM (Fluka). Control glands of all stages were treated identically except that no sodium glycerophophate was added to the incubation medium. Ultrathin sections were cut with glass knives on a Porter-Blum MT-2 microtome, stained with lead citrate (*8*), and examined under an electron microscope (Philips 200).

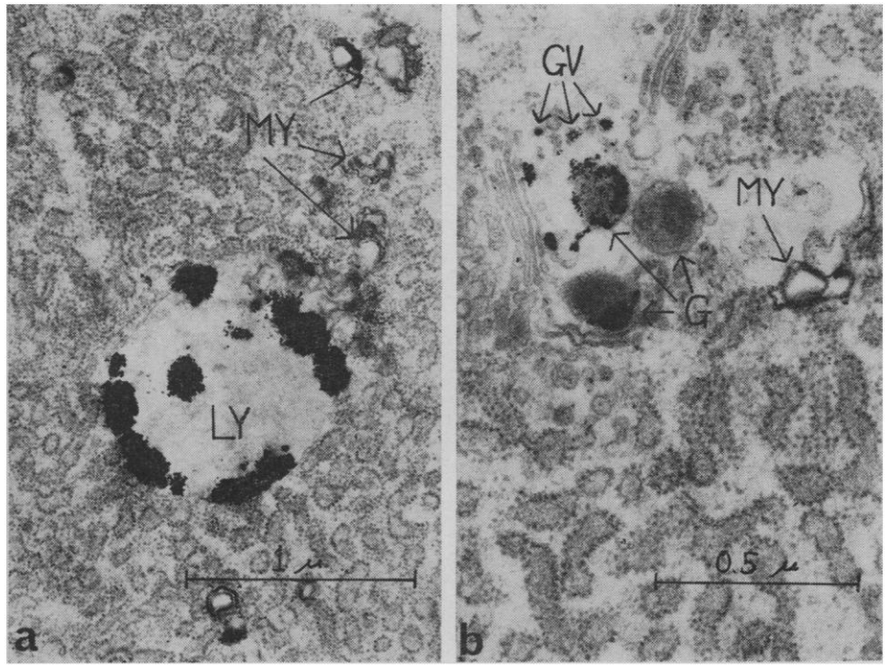

Fig. 1. (*a*) Lysosome (*LY*) and (*b*) small electron-opaque granules (*G*) with acid phosphatase activity in salivary-gland cells of last-instar larvae; *GV*, Golgi vacuoles; *MY*, myelin-like figures.

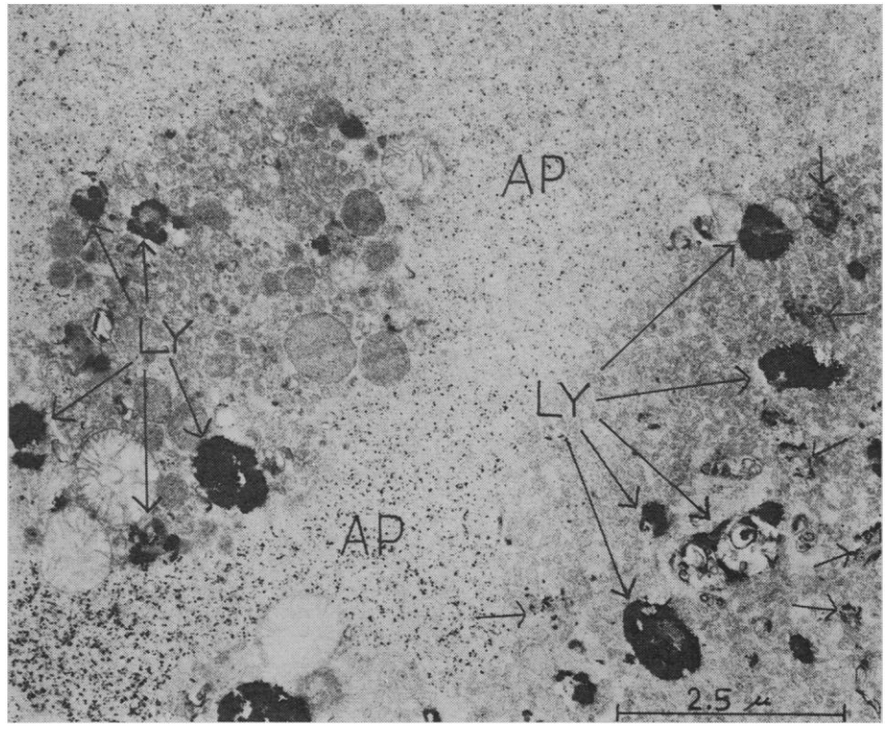

Fig. 2. Free (unbound) acid phosphatase activity (*AP*) in degenerated cell parts, and lysosome-bound acid phosphatase activity (*LY*) in nondegenerated cell parts of a salivary gland of a young pupa; *arrows*, Golgi bodies with strong acid phosphatase activity.

In salivary-gland cells of older last-instar larvae and prepupae the reaction product of acid phosphatase was always associated with one of several cytoplasmic structures. The most remarkable of these structures were bodies 0.5 to more than 1 μ in diameter that closely resembled vertebrate lysosomes (Fig. 1*a*); they were slightly opaque and frequently contained fragments of myelin-like figures. Without detailed discussion, in this report we term these structures lysosomes. A second type of structure containing the reaction product of acid phosphatase was much smaller and more opaque (Fig. 1*b*); it always occurred near Golgi bodies. Golgi vacuoles also have been frequently observed to contain reaction products of acid phosphatase.

In *Chironomus tentans*, breakdown of the salivary glands begins only at the end of the larval-pupal molt. Glands of very young pupae may consist of cells that are in the process of degeneration, alternating with cells not yet degenerating. Even within single cells, degenerating areas may alternate with not-yet-degenerating areas. In cells or cellular areas that were not yet degenerating, we found acid phosphatase activity only in structures similar to and probably identical with those described for younger larvae (Fig. 2). The lysosomes were polymorphic and of rather irregular shape. The amount of acid phosphatase reaction product bound to lysosomes and Golgi bodies (Fig. 2) seemed to be considerably larger in pupae than in younger larvae. In degenerating cells, or cell parts, lysosomes and Golgi bodies were no longer present; acid phosphatase reaction product was then freely distributed in the cytoplasm.

Our results indicate that the occurrence of reaction product of free acid phosphatase is not an artifact, and that in fact our slides show the true distribution of the enzyme at the moment of fixation. This conclusion is based on the facts that (i) free acid phosphatase has never been found in larvae

before cell breakdown, and (ii) in individual cells and slides there may be both: areas having free acid phosphatase and areas having intact lysosomes. Our evidence suggests that acid phosphatase is liberated at the moment of cell breakdown by rupture of the lysosomes.

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Codominance of Visual Pigments in Hybrid Fishes

Abstract. *Visual pigments of lake char and brook char (Salmonidae) are based on two different proteins. Both proteins are present in first-generation hybrids between these species and they segregate in second-generation and backcross hybrids, as expected of a single-factor difference. This first genetic study suggests that shifts observed in the absorption spectra maxima of visual pigments are related to substitutions of amino acids in the visual proteins.*

The visual pigments are proteins conjugated with a carotenoid prosthetic group known variously as vitamin A aldehyde, retinene, or retinal (*1*). The absorption spectra of visual pigments depend in part upon the prosthetic group, which is either retinene-1 or retinene-2. Either retinene can combine with the appropriate protein (opsin) to form a visual pigment. Hence, for every opsin, there is a pair of visual pigments; a mathematical relation between the wavelengths of maximum absorbance (λ_{max}) of such pairs has been described by Dartnall and Lythgoe (*2*). Many spectrally distinct visual pigments have been examined, and two series have been described, one based on retinene-1 (*2*) and another on retinene-2 (*2*, *3*). The differences within each series are believed to depend on variations in the structure of the opsins (*4*). However, little is known about opsins as proteins because their low concentration and insolubility make standard biochemical study difficult (*5*).

The visual pigments of the trout-like fishes called chars (family Salmonidae, genus *Salvelinus*) have recently been investigated by the method of partial bleaching (*6*). The brook or speckled char, *Salvelinus fontinalis* (Mitchill), has a retinene-1 pigment with a λ_{max} of 503 ± 1 mμ. The lake char, *S. namaycush* (Walbaum), has a different retinene-1 pigment with a λ_{max} of 512 ± 1 mμ. In addition, they also have the corresponding retinene-2 pigments. Fertile hybrids between these species can be produced by artificial fertilization and these hybrids are called "splake" (from speckled × lake). The first-generation hybrids (F_1 splake) appeared to have both parental retinene-1 pigments, together with the corresponding retinene-2 pigments (*6*). This evidence suggested that these pigments are inherited with codominance, in the same way as human M,N blood antigens are, but the hypothesis was uncertain because the visual pigments were studied only in retinal extracts from several animals and not in individual fish. Furthermore, the analysis of four visual pigments mixed together in the same extract is extremely difficult.

We have obtained additional material, including F_1 splake, second-generation hybrids (F_2 splake), and the backcross progeny of F_1 splake and brook char parents (*7*). The retinas of individual dark-adapted fish were frozen in 4-percent potassium alum solution and then stored at −20°C until used. Each opsin can be represented in two forms (as the retinene-1 and retinene-2 pigments), but the analytic difficulty this presents was avoided by bleaching the visual pigments and then regenerating them in the form of the retinene-1 pigment only (*8*). The frozen retinas were thawed, centrifuged to remove the alum solution, and washed twice with distilled water and once with 0.15*M* phosphate buffer (*p*H 6.5). They were extracted with 0.5 ml of freshly prepared 2 percent digitonin and treated with high-frequency sound to disrupt the visual cells. After centrifugation the supernatant extract was bleached exhaustively with orange light (610 mμ), which is not absorbed by the products of bleaching. To the bleached extract was added 0.02 ml of a solution of 11-*cis* retinene-1 in digitonin (*9*), and regeneration proceeded in darkness for 2 hours at 25°C. The regenerated extract was mixed with 0.02 ml of 0.8*M* neutral hydroxylamine solution to inactivate any excess retinene and was buffered with 0.05 ml of saturated sodium borate (final *p*H, 8.3 to 8.8).

Table 1. Frequency distribution of opsins in parental and hybrid chars. Each opsin is represented as the retinene-1 pigment.

Number of fish		
503_1 pigment	$503_1 + 512_1$	512_1 pigment
	Brook char	
7	0	0
	Lake char	
0	0	19
	F_1 splake	
0	12	0
	F_2 splake	
4	8	3
	Backcross (F_1 × brook char)	
8	7	0

The extracts were analyzed by the method of partial bleaching (*10*). In every experiment the photosensitive pigment was bleached in three stages (Fig. 1). The difference spectra of the regenerated retinene-1 pigments of both brook and lake char are identical to the difference spectra of the retinene-1 pigments obtained in separate experiments performed without prior bleaching and regeneration (Fig. 2). This shows that 11-*cis* retinene-1 is the visual isomer in these species. The experiments with retinal extracts of hybrid fish were all performed in the same way. The procedure gives three criteria for determining whether each extract contains a mixture of the two retinene-1 pigments (λ_{max} 503 and 512 mμ), or either pigment alone. As a consequence of the difference in λ_{max} of these pigments, the 512_1 pigment is more sensitive to red light. An exposure to red light bleaches extracts of