Table 1. Distribution of chromosome counts.

Cell line (RMPI No.)	Chromosome counts			Total cells	Tetraploidy* (%)
	45	46	47		
1608 (XYY)		9	92	101	0.7
1608 (XYY)	1	1	48	50	0.6
1638 (XXY)	2	18	80	100	1.0

* Determined from a sample of 600 to 800 metaphases under low-power optics.

1968. This culture, designated RPMI cell line No. 1638, has subsequently been divided every few days; portions of cells are being preserved in our cell bank. The cultured cells revealed a predominant karyotype 47 XXY chromosome constitution (Table 1).

The theoretical importance of maintaining permanent human cell lines with various chromosomal defects is self-evident. It remains to be seen whether or not critical biochemical expressions of such defects are retained by the cultured cells. Evidence in support of this hope has been provided by the continuing production of a Bence Jones protein by a lymphocytoid cell line derived from a patient with multiple myeloma (*8*). We are interested in establishing permanent cell lines from persons with genetic defects. Our laboratory is equipped to grow gram and kilogram amounts of these cell lines (*9*).

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18 November 1968; revised 24 January 1969

Protein Digestion in Isolated Lysosomes Inhibited by Intralysosomal Trypan Blue

Abstract. *Control rats and rats treated with subcutaneous trypan blue were injected intravenously with denatured albumin-I^{125}. Lysosome-rich fractions of their livers, when incubated at 22°C in osmotically protected medium (pH 7.4), retained their capacity to digest albumin-I^{125}. The rate of digestion was lower in suspensions prepared from rats treated with trypan blue than in control suspensions, but rates of lysosome breakage were not different. These results and other experimental evidence suggest that trypan blue concentrated within lysosomes can inhibit intralysosomal digestion, probably by inhibition of lysosomal proteinases.*

There is now evidence that the principal function of lysosomes is the intracellular digestion of macromolecules (*1*). In endocytic cells this process should be uniquely susceptible to modification by foreign compounds, and De Duve (*2*) has recently summarized the different forms such modification might take. One possibility is a reduced digestive capacity caused by uptake of enzyme inhibitors, and such a mechanism of action has been proposed for trypan blue (*3*) on the basis of its uptake into lysosomes and its ability to inhibit lysosomal enzymes in vitro (*4*). The experiments described have been undertaken to test this hypothesis.

Mego *et al.* (*5*) have demonstrated that after intravenous injection into mice formaldehyde-treated albumin-I^{131} is taken up into the liver heterolysosomes. They showed further that when a suspension of the resulting albumin-laden lysosomes was incubated at 37°C, in osmotically protected medium, degradation of their contained protein continued, with release of labeled iodotyrosine. In the work reported here, similar methods have been used to study the effect of trypan blue on intralysosomal digestion of protein. In our experiments male Wistar rats (270 to 310 g) were given an intravenous injection of denatured albumin-I^{125} (2.5 mg/kg in 0.1*M* saline). The labeled albumin was prepared by the method of McConaghey and Dixon (*6*) and denatured as described by Mego *et al.* (*5*).

Some rats received a subcutaneous injection of purified trypan blue (75 mg/kg) 24 hours before the albumin. Rats were killed 0.5 hours after injection with albumin, and their livers were removed immediately and homogenized in ice-cold 0.25*M* sucrose (10 ml per gram of liver) with a Teflon-on-glass homogenizer. After removal of cell debris and nuclei by low-speed centrifugation (1100*g* for 10 minutes) a large granule fraction containing lysosomes was sedimented by centrifugation at 16,500*g* for 20 minutes. This fraction was carefuly resuspended in 0.01*M* tris buffer (*p*H 7.4), containing 0.25*M* sucrose (5 ml per gram of liver), and immediately incubated at 22°C, the temperature being selected in order to reduce injury to the lysosomes during incubation. Samples were removed at intervals of up to 2 hours, placed in trichloroacetic acid (TCA) (final concentration 10 percent), and centrifuged to remove precipitated protein. This procedure served to precipitate the labeled albumin while allowing the products of digestion (iodotyrosine-I^{125}) to remain in solution. The supernatants were therefore decanted and counted in an automatic gamma well counter (Nuclear-Chicago Gammatic).

Seven experiments were performed in which the rate of digestion in a granule suspension prepared from a rat treated with both trypan blue and albumin was compared with that in a suspension prepared at the same time from a rat that had received albumin alone. In every case the rate was lower in granules from rats treated with the dye, a result that is consistent with the role of trypan blue as an intralysosomal inhibitor. Figure 1 shows the results of a typical experiment.

Conditions known to disrupt rat liver lysosomes, such as the absence of an adequate protective medium (that is, incubation in buffer only), the addition of 0.1 percent Triton X-100, or freezing and thawing the suspension prior to incubation, each abolished the breakdown of albumin. Furthermore, the addition of trypan blue in high concentration (20 mg/ml) to a control suspension did not decrease the rate of the breakdown of albumin, compared with that of controls. These experiments establish the site of the proteolysis illustrated by Fig. 1 to be intralysosomal and show that the dye must be concentrated within lysosomes to exert an effect on intralysosomal digestion. There is no reason to suppose that these wholly intralysosomal events would be modified in any way by the

other organelles, such as mitochondria, which are undoubtedly present in the suspensions.

It is possible, in addition to any direct action on lysosomal enzymes, that concentration of trypan blue within lysosomes may accelerate the disruption of the digestive vacuoles. This breakage, as shown with Triton X-100 and unprotective medium, would result in an overall decrease in the rate of protein hydrolysis, resulting from dilution of enzymes and substrate into the incubation medium. The release of albumin and of acid phosphatase from granules under our experimental conditions was therefore measured.

The level of undigested albumin released into the suspension was estimated by determining the nonsedimentable radioactivity that was insoluble in TCA. Samples were removed from granule suspensions at various times during incubation, immediately cooled to 0°C and centrifuged at 20,000*g* for 20 minutes at 4°C. The supernatants were decanted into tubes containing TCA (final concentration 10 percent) and counted to obtain the total nonsedimentable radioactivity. They were again centrifuged to remove the TCA-insoluble protein, and the supernatant was decanted to determine the radioactivity that was insoluble in TCA. The latter counts were subtracted from the total to obtain the TCA-insoluble, nonsedimentable radioactivity. Preliminary experiments had shown that, when granule suspensions were incubated at 22°C in 0.01*M* tris buffer (*p*H 7.4) without protective sucrose, 90 percent of the radioactivity was recovered as nonsedimentable albumin in a high-speed supernatant. Thus the level of nonsedimentable albumin reflects the extent of rupture of particles containing labeled albumin.

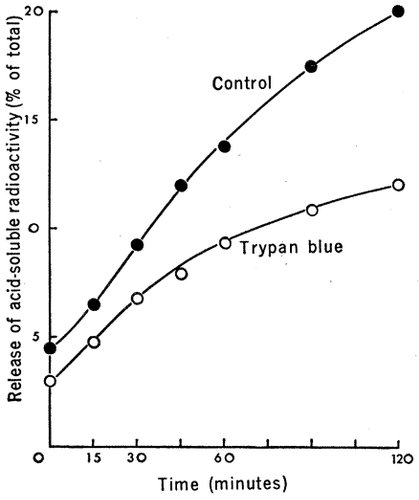

Fig. 1. Effect of intralysosomal trypan blue on the release of acid-soluble radioactivity during the incubation of rat liver heterolysosomes laden with radioiodinated albumin. A rat was injected subcutaneously with trypan blue (75 mg/kg). Twenty-four hours later this rat and an uninjected control of the same weight were injected intravenously with albumin I^{125} (2.5 mg/kg). After 30 minutes the livers were removed and homogenized. Particles separating between 1100*g* for 10 minutes and 16,500*g* for 20 minutes were suspended in tris-acetate buffer (*p*H 7.4) containing 0.25*M* sucrose and incubated at 22°C. Samples were withdrawn and assayed for TCA-soluble radioactivity. The percentage of total radioactivity becoming TCA-soluble in 120 minutes was lower in granules from animals treated with trypan blue than in their paired controls (range, 26 to 40 percent lower in seven experiments).

The results obtained with protective sucrose show there is little difference in the release of either albumin (Fig. 2) or acid phosphatase (Fig. 3) between suspensions prepared from animals treated with dye or control animals. It may therefore be concluded that the stability of the granules under the incubation conditions studied is not affected by the presence or absence of intralysosomal trypan blue. The differences between Figs. 2 and 3 may reflect the inevitable presence in the granule suspension of phagosomes containing albumin, but no acid phosphatase, and of lysosomes containing no albumin. Also, acid phosphatase release from broken lysosomes is probably incomplete, so that levels of nonsedimentable enzyme will underestimate the extent of lysosome rupture. Further, the possibility cannot be excluded that release of partially digested albumin from intact lysosomes makes some contribution to the levels of TCA-insoluble, nonsedimentable radioactivity plotted in Fig. 2.

Our results clearly demonstrate that concentration of trypan blue within the vacuolar system is able to reduce the rate of digestion of denatured albumin-I^{125} within lysosomes. When similar experiments were carried out with a suspension of lysosomes prepared from a rat injected with carbon (160 mg/kg) 24 hours before injection of labeled albumin, some increase in the rate of iodotyrosine-I^{125} release was observed. Therefore the concentration of a nonspecific foreign compound within digestive vacuoles does not depress the breakdown of bovine serum albumin-I^{125}. Hence, we have evidence to suggest that trypan blue is able to inhibit intralysosomal digestion of a protein, presumably by inhibiting one or more of the lysosomal proteinases. It is possible that a similar action is exerted on the other acid hydrolases of rat liver lysosomes. Histochemical evidence (*7*) supports the possibility of acid phosphatase inhibition. In a previous paper (*3*) we proposed that inhibition of acid hydrolases in the lysosomes of the rat yolk-sac during the period when this organ is solely responsible for embryonic nutrition could be induced by trypan blue. This might cause a temporary disturbance of embryonic nutrition with resulting congenital malformation. The present findings—although carried

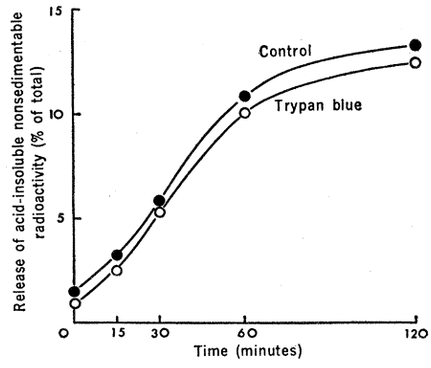

Fig. 2. Release of acid-insoluble, nonsedimentable radioactivity from the lysosomal suspensions prepared and incubated as described in Fig. 1.

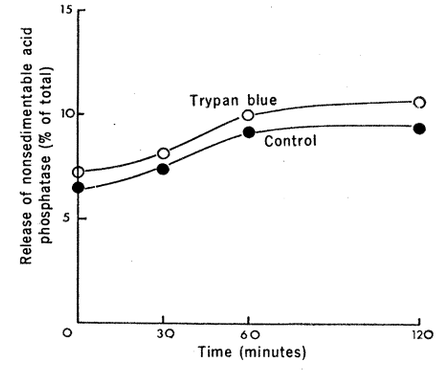

Fig. 3. Release of nonsedimentable acid phosphatase in the experiment described in Fig. 1. Samples were removed from the suspension and centrifuged at 20,000*g* for 20 minutes and the acid phosphatase activity of the supernatant was determined. Acid phosphatase was assayed at 37°C in acetate buffer (*p*H 4) and with $10^{-2}M$ *p*-nitrophenyl phosphate used as substrate. The total activity in each sample was determined by parallel determinations including 0.1 percent Triton X-100 in the 22°C incubation. Nonsedimentable acid phosphatase is expressed as percent of total activity in each sample.

out on rat liver lysosomes for experimental convenience—provide in vitro evidence consistent with such a suggestion.

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Cell Death during Early Morphogenesis: Parallels between Insect Limb and Vertebrate Limb Development

Abstract. *The complex jointed leg of the adult fly is derived, in the pupal stage, from a simple lobed sac of cells. The gross morphological changes that result in adult shape are effected by a combination of differential cell growth and the programmed death of a large number of imaginal disc cells. Events are closely similar to those occurring in digit formation and limb contouring during vertebrate morphogenesis. In both cases phagocytic blood cells are intimately involved.*

During the development of the vertebrate limb, digit formation is accompanied by programmed cell death, and areas of necrosing cells appear at specific sites and times (*1*). The death and resorption of these cells contribute to the morphogenetic movements which model not only the digits (Fig. 1A) but also the contours of the thigh and upper arm. In the duck the distribution of such areas of necrosis is correlated with the degree of webbing (*2, 3*). Between digits one and two, which in the fully formed foot are not connected by webbing, the interdigital zone of the footplate has V-shaped wedges of necrosis extending from the distal margin and involving some ectodermal cells and a high percentage of mesenchymal cells. In contrast, between digits two and three, and three and four, which are webbed in the adult, only shallow zones of necrosis occur. Only the distal regions of the interdigital tissue degenerate, the rest remaining as web. Inhibition of necrosis by injection of Janus green results in high frequency of syndactylism and in an absence of interdigital macrophages (*4*).

In limb development of *Sarcophaga bullata*, the flesh fly, a remarkably similar sequence of events occurs (Fig. 1B). The adult fly leg is composed of coxa, trochanter, femur, tibia, tarsus (composed of five tarsal segments), and a terminal pretarsus; the latter is composed of two claws and two footpads, or pulvilli, which the fly uses to adhere to the substratum. The tarsal segments are separated by joints that are deeply indented, and the two pulvilli of the pretarsus are also separated as far as their bases. Yet these structures are derived, at the beginning of adult development, from a simple sac of epidermal cells which follow the outlines of, and are closely apposed to, the pupal cuticle which they have themselves recently secreted. This simple sac of cells, together with the adhering pupal cuticle, is slightly lobed at the level of the future tarsal joints, and also very slightly lobed distally (*5*).

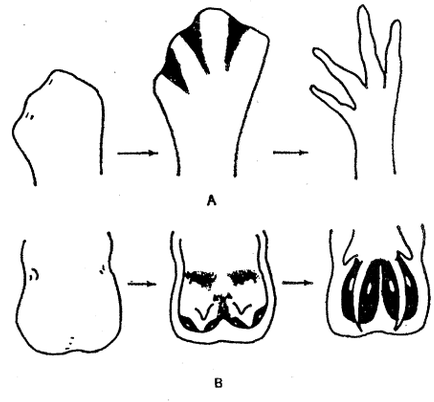

Fig. 1. Areas of cell necrosis in vertebrate and insect limbs during morphogenesis. (A) Diagram of relation between cell necrosis and digit formation in the chick (*3*). (B) Diagram of relation between cell necrosis and pulvillar formation in the fly. Areas of cell death denoted by the lightly shaded area; rapidly growing giant dorsal cells, black; unshaded nuclei, white.

The leg is present in the larval fly as an imaginal disc. At pupation, the pupal cuticle is secreted by the epidermal cells, and phagocytic hemocytes, gorged with larval tissue fragments, are forced into the leg as it is extended with hemolymph. Tracheoles, also epidermal, are present, and nerves extend as far as, but not into, the pretarsus. Since the tarsal segments are devoid of muscles in the adult, no mesodermal elements exist there other than the hemocytes.

Within 24 hours after pupation of the larval fly, the leg epidermis is withdrawn from the pupal cuticle, and extensive cell division is seen. By this time, certain cells can be distinguished by their larger nuclei and polytene chromosomes; these include five cells that migrate in file to form the distal region of each claw, two cells that will form basal plates, and four cells that will reach giant proportions and will each be responsible for the secretion of half the cuticle of the dorsal surface of each footpad (*6*). On the tarsal segments, future trichogen (hair) and tormogen (socket) cell nuclei are evident, also showing a low degree of polyteny. For those cells that will become large and have polytene chromosomes, increase in cell size and DNA replication continues from day 2 through into day 4 (*7*). Extensive cell division occurs ventrally in the pretarsus during day 2 and into day 3. The products of these cell divisions differentiate, during day 3, into tenent hair cells, responsible for forming the cuticular hairs of the ventral adhesive surface of each of the two pulvilli.

During days 3 and 4 there is virtually no cell division in the leg. This marks the period of cell growth, cell differentiation, and cell movement, bringing about the gross morphological changes that transform the simple lobed structures of a late day 2 pupa into the well-contoured adult form that is established by late day 4. It now appears that this same period is marked by dramatic cell death. Tenent cells break down later at day 9 to 10, and giant footpad cells break down at the time of their emergence. This is not surprising for