EFFECTS OF HYPERTHERMIA ON *TETRAHYMENA*

I. *Localization of Acid Hydrolases and Changes in Cell Ultrastructure*

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SUMMARY

The effects of hypothermia on *Tetrahymena pyriformis* (E) were studied. Protein synthesis was greatly inhibited at elevated temperatures (33.5–34°C), although the rate of protein turnover was not initially greater than in controls. There was no evidence that cellular lesions induced by temperature shock resulted from a solubilization of lysosomal enzymes. More than 80 % of both acid phosphatase and acid proteinase activity remained particle-bound, even in cells incubated at 34°C for prolonged periods. Ultrastructural studies revealed gross lesions in all types of cell organelles. These included a loss of tubules from mitochondria, disappearance of the rough endoplasmic reticulum, nuclear abnormalities, and a swelling of bodies tentatively identified as peroxisomes or primary lysosomes. Autophagic vacuoles which contained considerable membranous material seemed to accumulate during treatment, and membranous whorls and smooth-walled vesicles became quite common in the cytoplasm. Our studies are consistent with the suggestion [28] that, supraoptimal temperatures affect phospholipid-containing membranes in *Tetrahymena*.

The ciliated protozoan, *Tetrahymena pyriformis*, has been widely used in studies dealing with synchronized cell division. Synchronized division is generally induced by a series of repetitive heat shocks, in which the organism is periodically exposed to an elevated temperature which, if maintained, would eventually be lethal. It has been reported that the elevated temperature inhibits protein [3, 20] and RNA synthesis [19], oxidative phosphorylation [25, 26], and development of the oral region [14]. Rosenbaum et al. [28] have noted gross abnormalities in cells incubated at elevated temperatures, but also found that cells could be grown at temperatures that were otherwise lethal, provided that the culture medium was supplemented with phospholipids. It was suggested that all of the observed effects of hyperthermia on this ciliate could result from a derangement of normal membrane structure and function. Recent work

on bacteria has also led to the suggestion that metabolic lesions induced by hyperthermia may result more from damage to cellular membranes than from damage to individual enzymes (cf [2]). In line with this, it has been reported [5, 6] that 2-amino ethyl phosphonic acid, a component of cellular membranes in *Tetrahymena* [29], is released upon incubation of cells at elevated temperatures.

This paper deals with the effects of hyperthermia on the integrity of lysosomes and other cell organelles. The lysosome has been widely implicated in cellular degradative processes, and it has often been suggested that cellular damage and death resulting from environmental stress may be due to a bursting of these organelles, with subsequent release of hydrolytic enzymes into the cytoplasm [1, 7]. Biochemical data presented here indicate that the lesions that occur when *Tetrahymena* is subjected to long periods of hyperthermia do not result from a solubilization of lysosomal enzymes. However, ultrastruc-

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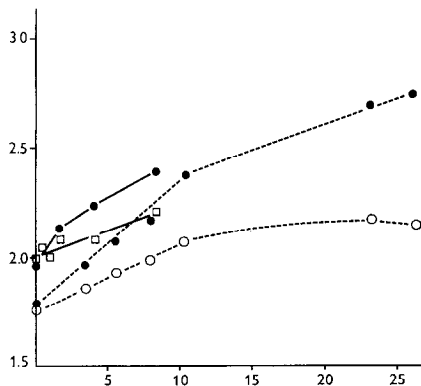[1] Present address: Faculty of Biological Sciences, Southern Illinois University, Edwardsville, Ill. 62025 USA.

Fig. 1. Culture growth at 28, 33, 34°C. Log phase cultures, growing in a water bath-shaker at 28°C, were transferred to 33°C (○–○), 34°C (□–□) or maintained at 28°C (●–●). --- and —— represent separate experiments. *Abscissa:* Time, h; *ordinate:* μg protein/ml culture, log.

Fig. 2. Protein turnover at 33.5°C. Cultures were grown for 24 h in peptone–liver fraction containing 2 μc of ^{14}C-serine. Turnover in washed cells (a) was tested by harvesting and washing cells in sterile buffer, and incubating cell suspensions at 28 or 33.5°C. At the times indicated, aliquots were removed and cell protein was tested for radioactivity. Turnover was measured in cultures (b) by diluting growing cultures with 4.5 vol peptone supplemented with enough ^{12}C-serine to give a 1000-fold dilution over the original amount of ^{14}C-serine added. *Abscissa:* Time, h; *ordinate:* radioactivity, % of original activity. ○–○, controls; ●–●, cells incubated at 33.5°C.

tural evidence reveals that most other cell organelles and inclusions are severely damaged by such treatment.

MATERIALS AND METHODS

Cultures of *T. pyriformis* (E) were grown on proteose-peptone-liver fraction, as described previously [21]. For heat shock studies, log phase cultures were transferred to a water bath-shaker at 33–33.5°C. (In initial experiments, the test temperature was 34°C, but this frequently was lethal after several hours.) Cells were collected at various times after transfer, washed twice in an inorganic phosphate-buffered medium, and once in either 0.25 M or 0.33 M sucrose. They were then disrupted in an ice-cold sucrose solution of the same molarity in a smooth-walled glass homogenizer with a teflon pestle. Homogenates were centrifuged for 15 min at 27,000 g in a refrigerated centrifuge (Sorvall RC-2). The supernatant fluid was decanted and the pellet (usually 1 cc or less) was resuspended in about 10 ml of sucrose. Both samples were recentrifuged at the same speed. Three fractions were thus obtained—a pellet (*P*), a supernatant (*S*), and a wash of the pellet (*W*). Under these conditions, about 83 % of the total acid phosphatase appeared in the pellet, a value quite similar to that obtained by Müller et al. [24], who used a different means of cell disruption.

Acid phosphatase was determined by the method of Torriani [31] at pH 5 using *p*-nitrophenyl phosphate (*p*NPP) as substrate. Attempts to determine free and latent activity generally gave inconsistent results. Cathepsin was determined at pH 3.6 by the method of Gianetto & de Duve [15], using haemoglobin as substrate. Both enzymes were tested at three levels. Triton X-100 was routinely included in the reaction mixture, at a concentration of 0.1 % (v/v). Details have been given previously [22]. Protein was determined by the method of Lowry [23] on material that was insoluble after digestion in 3 % perchloric acid for 15 min at 90 %. Crystalline bovine serum albumin was used as a standard. Portions of cul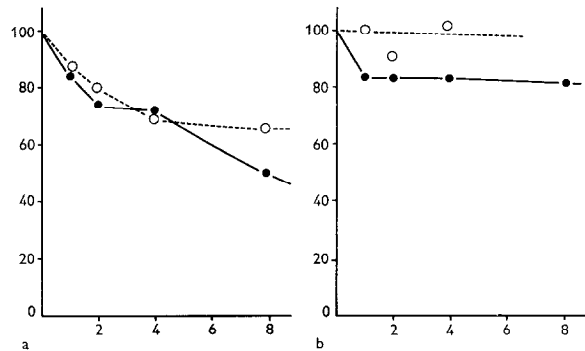ture that were to be tested for protein content were centrifuged, and the pellet of packed cells that remained was extracted with 50 % ethanol prior to digestion in perchloric acid.

Procedures for electron microscopy have been described in detail elsewhere [22]. Briefly, cells were fixed in phosphate-buffered glutaraldehyde, postfixed in osmium tetroxide, and embedded in Epon. Sections were stained with alkaline lead citrate [27] and examined with an RCA EMU3 electron microscope. Acid phosphatase was localized by reacting glutaraldehyde-fixed cells with Gomori's medium [16] for 15 min at room temperature. Control cells were incubated in the absence of substrate or in the presence of fluoride.

To prepare proteins for radioactive counting, cells were digested in 3 % perchloric acid at 90°C for 15 min. Precipitates were rinsed twice with perchloric acid, and then extracted twice with chloroform-methanol (3:1). Residues were dissolved in dilute NaOH, plated at infinite thinness on aluminium planchettes, and counted in a Nuclear-Chicago thin window gas flow counter. Values are the average of duplicate cell samples, each of which was plated in duplicate. A minimum of 1000 counts was recorded for each planchette.

RESULTS

Protein synthesis and turnover

Some initial studies were carried out to determine certain growth characteristics of *T. pyriformis* at elevated temperatures. Under our culture conditions, the critical temperature for this strain seems to be between 33.5 and 34°C.

Table 1. *Incorporation of amino acids into cell protein after various durations of incubation at 34°C*

Time (h)	Cpm/mg protein		Inhibition[a] (%)
	28°C	34°C	
0	238	164	32
0.5	—	126	49
1	—	101	59
2	239	97	60
4	258	96	61
8	163	107	56

[a] Based on average of 0, 2, and 4 h controls.

A growing culture of *Tetrahymena* was transferred to a water bath at 34°C, while another was maintained at 28°C. At the times indicated, aliquots of culture were mixed with 0.5 µc of uniformly-labeled ^{14}C-serine. Cells were incubated with isotope for a 20-min period at their respective temperature. Reactions were terminated by addition of perchloric acid.

In a few initial experiments, ciliates were incubated at the latter temperature. While some cultures survived for more than 24 h, others succumbed within 8 h, so in later experiments a temperature of 33.5°C was used for the hyperthermic shock.

Fig. 1 shows culture growth (in terms of protein) at 28°, 33°, and 34°C. The doubling time at 28°C is about 5 h (as determined by the initial slope of the curve with the dashed line). At 33°C, culture protein doubled in the first 10 h, but there was little further increase. At 34°C, the doubling time was greater than 13 h.

A second experiment tested the ability of cells to incorporate radioactive precursors into protein (table 1). In the first 20 min, uptake of tracer into cell protein was inhibited by about 30%. The inhibition increased to 60% by 1 h, and then remained at this level.

Table 2. *Localization of acid hydrolases in cells incubated at 33.5°C*

Time at 33.5°C (h)	Fraction	Acid phosphatase			Proteinase		
		Sp. act.	R.S.A.	In fraction (%)	Sp. act.	R.S.A.	In fraction (%)
0	H	19.3	(1.0)	—	1.2	(1.0)	—
	P	41.0	2.1	82.7	2.9	2.4	82
	S	6.85	0.35	13.6	0.48	0.90	13
	W	16.0	0.83	3.5	1.4	1.1	4
		(Recovery, 101%)			(Recovery, 119%)		
4	H	16.4	(1.0)	—	1.2	(1.0)	—
	P	33.6	2.1	84.3	2.1	1.8	84
	S	6.30	0.38	11.8	0.54	0.45	16
	W	9.45	0.58	4.0	—	—	0
		(Recovery, 125%)			(Recovery, 110%)		
8	H	14.7	(1.0)	—	1.1	(1.0)	—
	P	25.3	1.7	81.2	1.9	1.7	86–90
	S	5.32	0.36	14.0	0.26	0.24	9.7–10
	W	8.45	0.57	4.7	< 0.5	—	< 4
		(Recovery, 101%)			(Recovery, 96–98%)		
24	H	21.6	(1.0)	—	2.0	(1.0)	—
	P	41.2	1.9	83.5	4.4	2.2	83–90
	S	5.51	0.26	13.3	0.39	0.20	8.7–9.9
	W	24.6	1.1	3.4	< 0.7	—	< 8.7
		(Recovery, 103%)			(Recovery, 111–121%)		

Cells were incubated at 33.5°C for the times indicated. The specific activity of acid phosphatase is expressed as µmoles nitrophenol released $\times$ h$^{-1} \times$ mg protein^{-1}, and that of acid proteinase as mg of protein (bovine-serum albumin equivalents) released $\times$ h$^{-1} \times$ mg protein^{-1}. The relative specific activity refers to the specific activity of a given fraction as compared with that of the total homogenate (H). The percentage of activity in each fraction was calculated from the total activity recovered in the pellet (P), supernatant (S), and wash (W) fractions. The recovery compares the total activity in these fractions to that of the total homogenate.
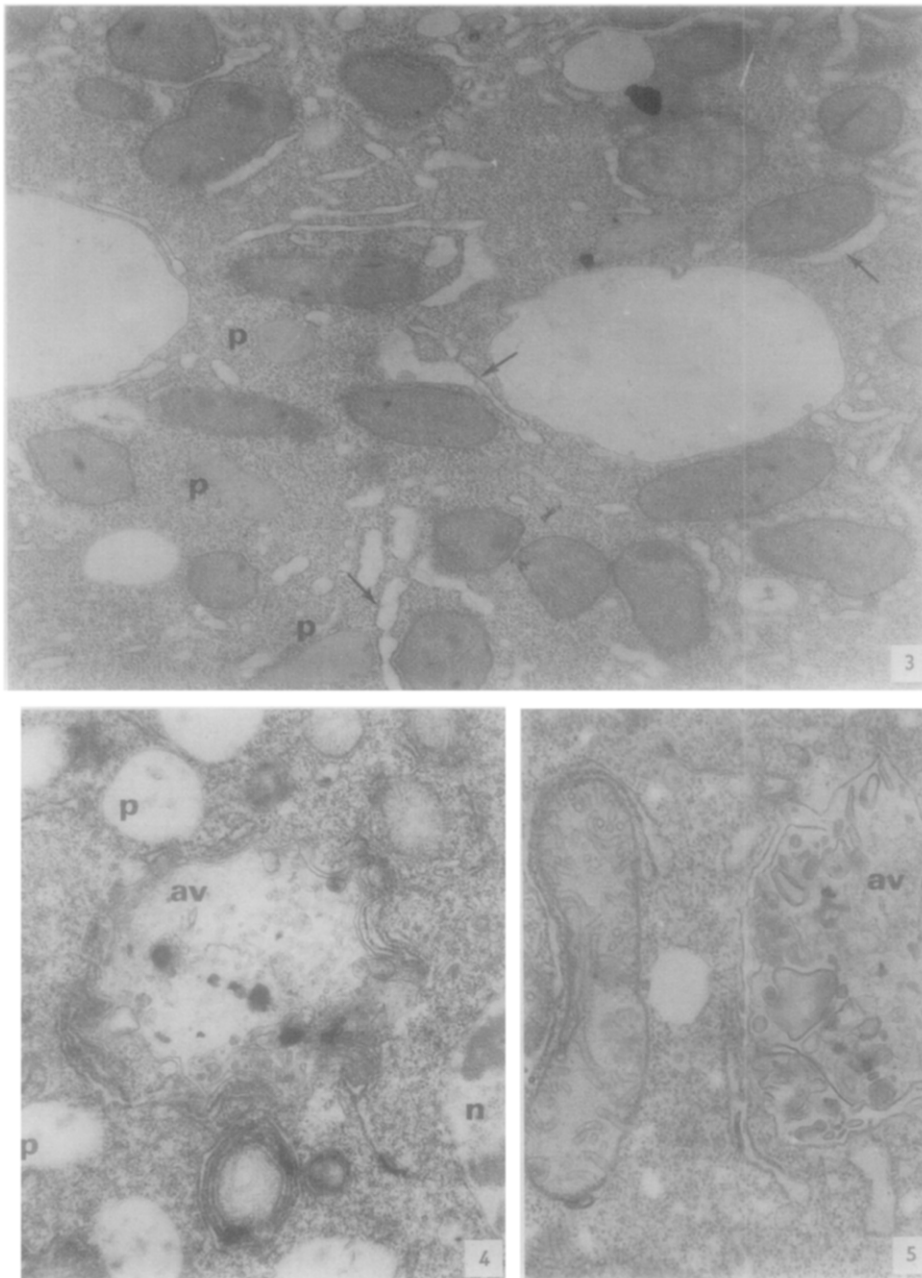
Fig. 3. Section through a normal cell, showing numerous mitochondria, rough endoplasmic reticulum (*arrows*) and organelles believed to be peroxisomes or primary lysosomes (*P*). × 15,000.
Figs 4, 5. Sections from cells incubated for 1 h at 34°C. Cells were tested for acid phosphatase by Gomori's procedure. Numerous mitochondrial tubules are seen in the autophagic vacuole (*AV*) in fig. 4. *N*, nucleus; *P*, peroxisome or primary lysosome. × 20,000 and 28,000.

In a third experiment, protein turnover was compared in normal and heat-shocked cells (fig. 2). To eliminate any possible differences resulting from lack of growth in the latter group, turnover was studied in both cultures and washed cell suspensions. Cultures were grown in the presence of ^{14}C-serine for 24 h, and then either transferred to fresh culture medium or washed

and transferred to inorganic medium. In the former case, there was a slight (17%) loss of tracer from protein during the 1st h at 33.5%, but no further loss after that. In washed cells, the rates of turnover were similar at the two temperatures except, perhaps, after a prolonged period of shock.

Localization of acid hydrolases

Some of the damage resulting from hyperthermia could result from destruction of lysosomes, with subsequent release of acid hydrolases into the cytoplasm [1, 7]. To determine if this occurred, we compared the localization of acid phosphatase and acid proteinase (cathepsin) in log phase cells with that of cells subjected to temperature shock for periods of 1 to 24 h. The results of a typical experiment are shown in table 2. It can be seen that there was little or no solubilization of either enzyme. About 83% of the acid phosphatase was readily sedimentable both in log phase cells and in cells subjected to 33.5 or 34°C for periods of up to 24 h. Müller [24] has obtained the same distribution using another means of cell disruption. In general, attempts to determine the degree of structural-linked latency were unsuccessful. However, in one experiment, the free activities for acid phosphatase were 39, 31, 31, and 29% of the total, respectively, for cells tested at 0, 1.25, 4, and 22 h after incubation at 34°C, suggesting that the permeability of the lysosomal membrane to substrate did not increase due to heat shock. There were no major changes in the specific activity of acid phosphatase during the course of the experiment.

Cathepsin activity behaved in a manner similar to that of acid phosphatase, in that there was no increase of enzyme in the soluble fraction of the cell. In fact, the percentage in the particulate fraction usually increased slightly. There generally seemed to be some increase in the specific activity of acid proteinase during the latter stages of heat shock, especially if these were prolonged to 34 h.

Several experiments similar to the one just discussed gave essentially the same results. About 83–85% of the acid phosphatase activity, and

80% or more of the cathepsin activity was readily sedimentable in homogenates from cells incubated at elevated temperatures for 0 to 25 h. (Fractionations were not done when the number of dead cells exceeded 1% or 2% of the total. In almost every case, only negligible numbers were observed.) These studies indicate that release of acid hydrolases into the soluble portion of the cell is not responsible for lesions resulting from prolonged hyperthermia. However, the possibility that damage to the lysosomal membrane may contribute to these lesions is not eliminated (see discussion).

Ultrastructural studies

Some preliminary studies on cell ultrastructure show that hyperthermia produces gross lesions in virtually all types of cell organelles. These include loss of tubules from mitochondria, virtual disappearance of rough endoplasmic reticulum (RER), swelling of unidentified organelles, abnormal nuclei which contain large inclusions, and general cell disruption.

Fig. 3 shows a portion of a control cell, showing normal mitochondria and organelles tentatively identified as peroxisomes, although they could be primary lysosomes (see also [22]). The mitochondrial tubules are generally well-packed, and many of the mitochondria are associated with a portion of RER in such cells. Autophagic vacuoles are common, but they differ from those seen in temperature-shocked cells.

Figs 4 and 5 show cells that were incubated for 1 h at 34°C (these were stained for acid phosphatase activity). In fig. 4, numerous mitochondrial tubules are present in the autophagic vacuole. This is of particular interest since many of the mitochondria become almost completely devoid of tubules if heat shock is continued (figs 8, 9). This figure also shows several partially evacuolated organelles which are probably peroxisomes or primary lysosomes. The lack of lead phosphate deposits suggests that they might be the former. Micrographs of cells incubated for periods of 0.5 to 5 h at 34°C reveal that these organelles rapidly become quite swollen in response to temperature shock, often becoming
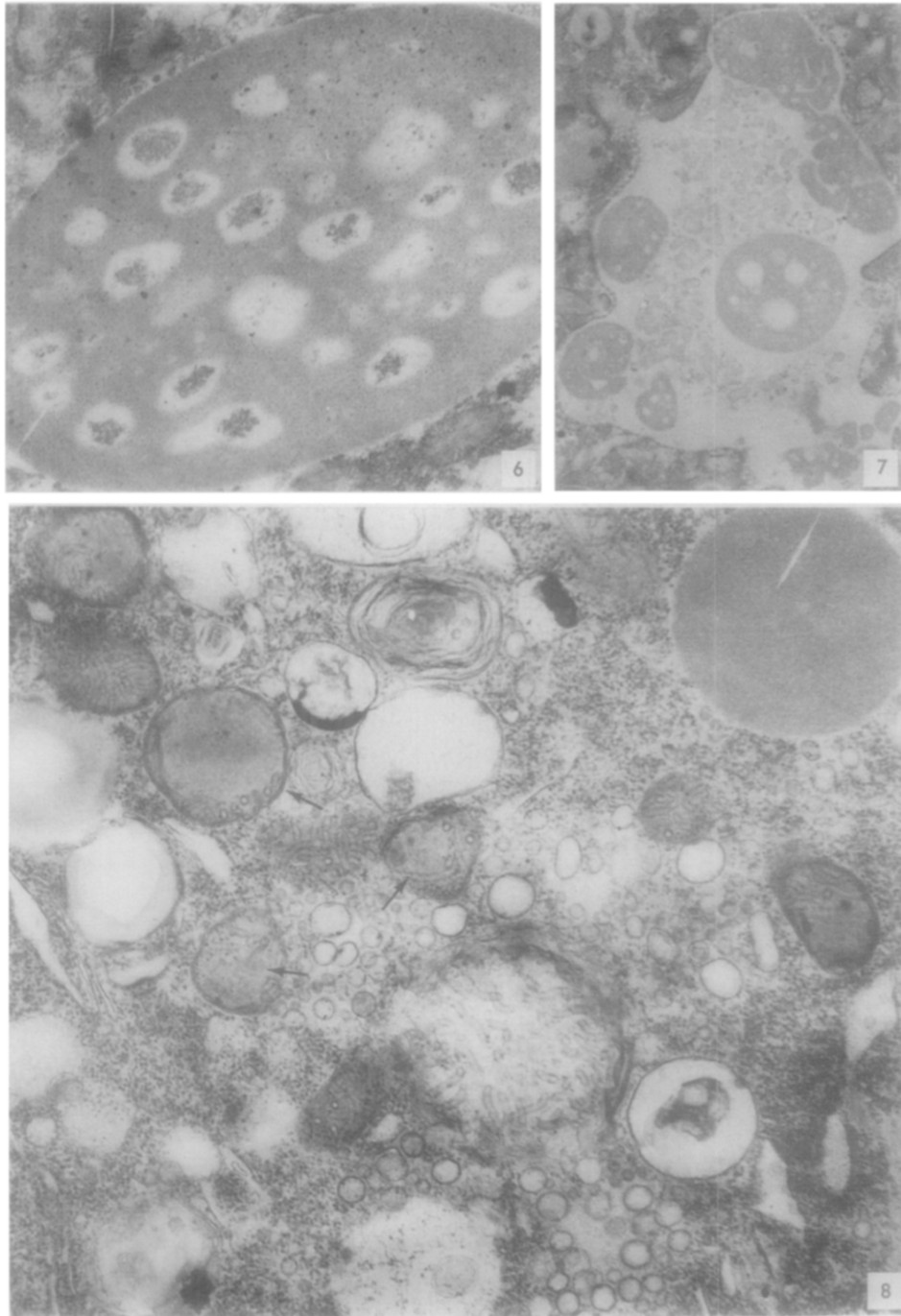
Figs 6–9. These are sections from cells incubated for 11.5 h at 34°C and stained for acid phosphatase. (*6*) Cytoplasmic body resembling those found in nuclei of cells subjected to prolonged hyperthermia. Note relative size of mitochondria. ×20,000; (*7*) Cell nucleus, showing large bodies which may have arisen from fusion of nucleoli. ×8,000; (*8, 9*) Several mitochondria (*arrows*) which are largely devoid of tubules are present. Also seen are membranous whorls and numerous smoothwalled vesicles. Several vacuoles in fig. 8 show acid phosphatase reaction product. ×23,000, 27,000.

Fig. 10. Section from a cell incubated for 11½ h at 34°C and tested for acid phosphatase activity. Several inclusions believed to be vacuolated lipid droplets are present (*L*). Reaction product is prominent in an autophagic vacuole. ×17,000.
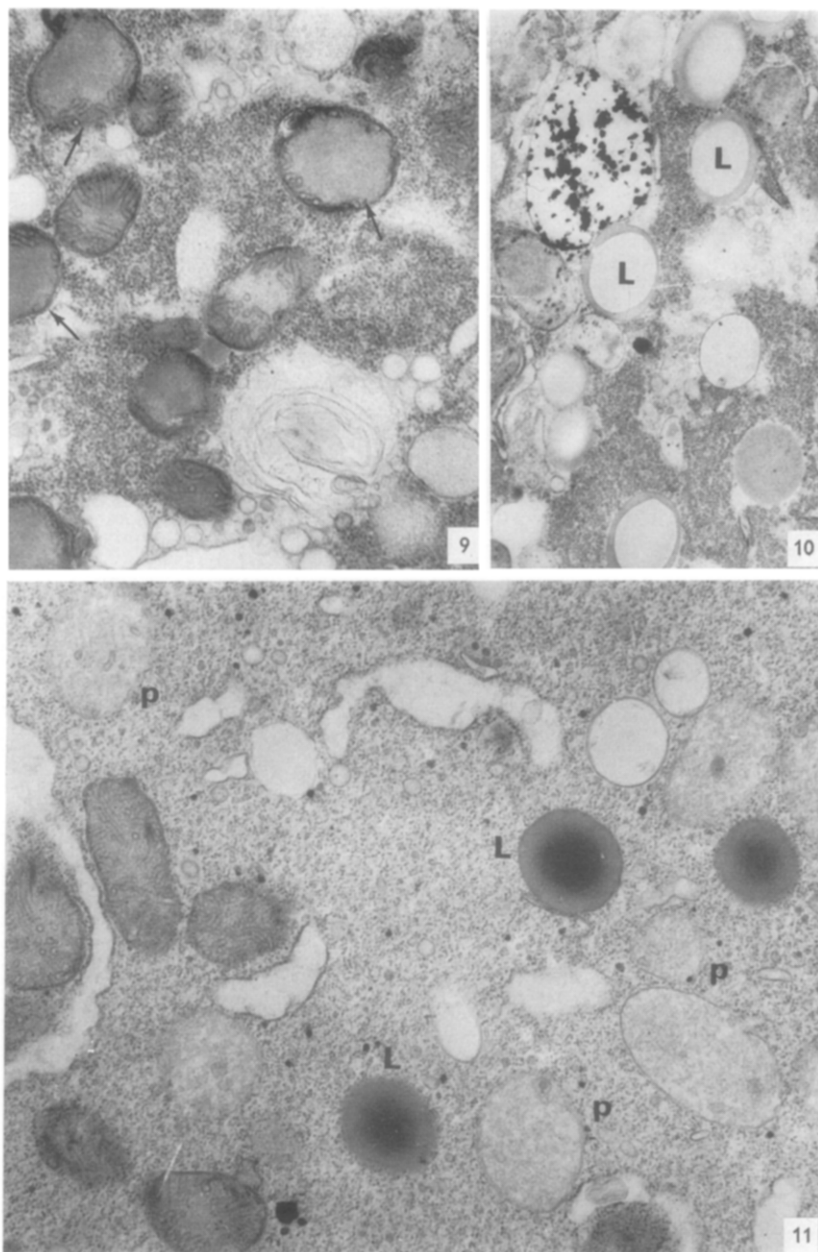
Fig. 11. Section from a cell incubated for 2 h at 34°C, again showing swollen appearance of organelles thought to be peroxisomes or primary lysosomes (*P*). These are normally smaller in diameter than mitochondria (see also fig. 12). Several lipid droplets (*L*) are also shown. × 15,000.

much greater in diameter than mitochondria (see also figs 11, 12). Numerous micrographs of normal and starved cells [22] have never revealed such an appearance, so it seems likely that the swelling is a result of temperature shock and not a fixation artifact.

Fig. 5 shows an autophagic vacuole which contains a considerable number of membranous remnants. There is an apparent loss of tubules from the large mitochondrion.

Fig. 7 shows a nucleus with several large inclusions. They become numerous in both the nucleus and cytoplasm in cells subjected to long periods of hyperthermia. Fig. 6 shows one of these which was found in the cytoplasm. While the nature of these bodies is uncertain, they may result from fusion of nucleoli, an event which has been observed in starved and stationary phase cells, and in cells subjected to short periods of temperature shock [4, 12]. In none of these
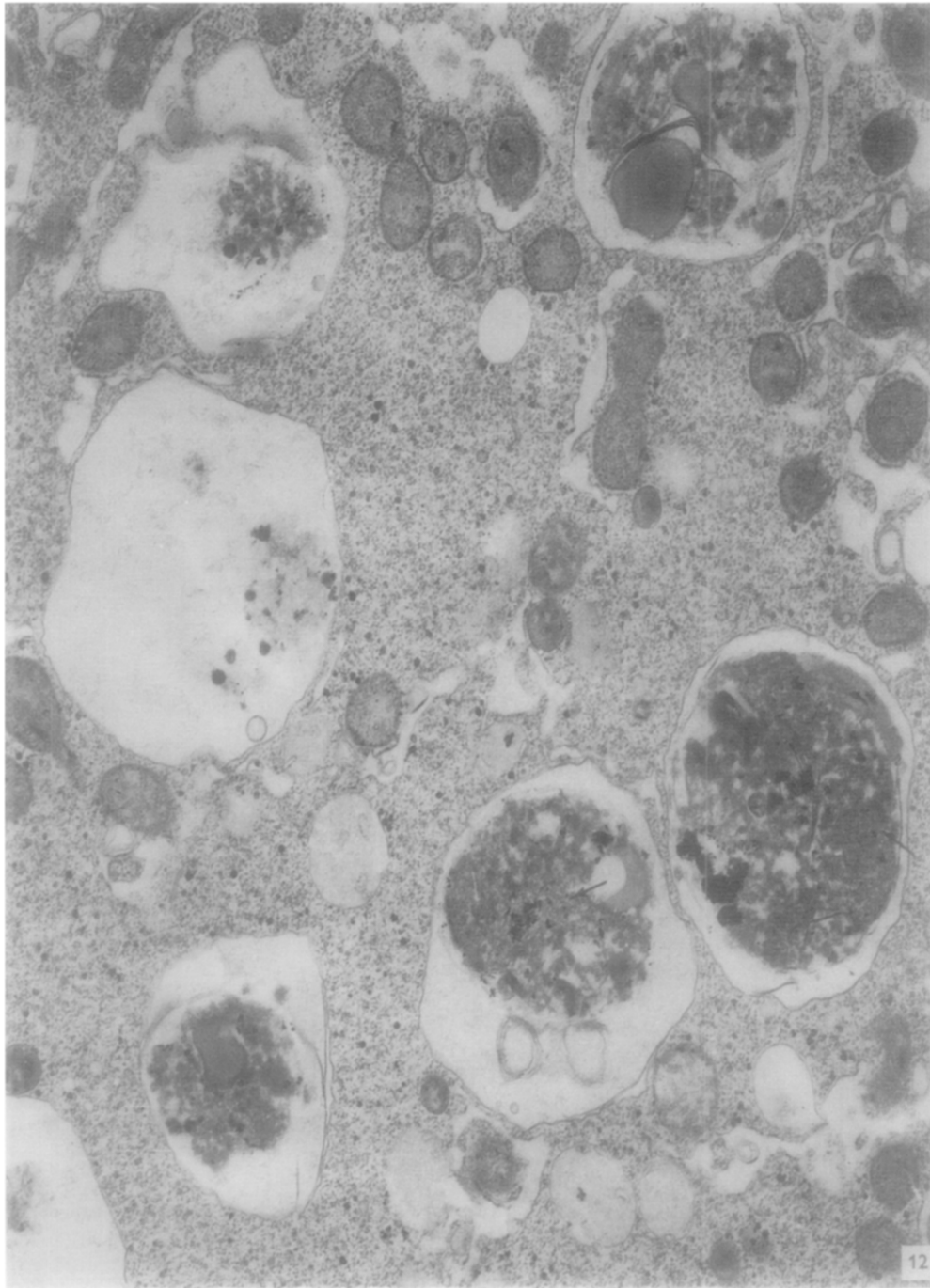
Fig. 12. Section from a cell incubated for 5 h at 34°C. Numerous autophagic vacuoles are present. Mito-chondrial remnants can be recognized in at least 2 of these *(arrows).* × 18,000.

cases, however, did the bodies resemble those shown here. The transfer of nucleolar-like bodies from the nucleus to the cytoplasm has been reported [4, 12, 22].

Figs 8 and 9 show sections of cells subjected to 34°C for 11 h. There is a general disruption of cell structure, with a virtual absence of RER, and numerous smooth-walled vesicles or tubules

are apparent throughout the cytoplasm. Many of the mitochondria are now almost devoid of tubules, with only a few of these structures present at the periphery. Membranous whorls such as the ones shown are numerous in the cytoplasm.

Autophagic vacuoles also may become abundant during temperature shock. Fig. 12, from a cell incubated for 5 h at 34°C, shows several large autophagic vacuoles. A considerable amount of membranous material, including mitochondrial remnants, is present in several of these. Formation of such vacuoles seems to be a general response to conditions that lead to the breakdown of cytoplasmic constituents, as they also become prominent in starved [22] and in stationary phase cells [11]. Lipid-like bodies, many of which are evacuolated, also become abundant as the hyperthermia progresses (figs 10, 11). Acid phosphatase reaction product is present in autophagic vacuoles in figs 8 and 10.

DISCUSSION

Many studies on microbial systems have indicated that cessation of growth at supraoptimal temperatures often results from the inhibition of a single enzyme. Growth can often be restored by addition of the end product of these reactions or by an appropriate cofactor (cf [18]). However, increasing evidence suggests that hyper- (or hypo)thermic lesions may result from a disruption of the integrity of various cellular membranes, with subsequent loss of function of enzymes or enzyme systems that are localized on these membranes (cf [2]). Rosenbaum et al. [28] have emphasized that most of the lesions observed in ciliates that have been maintained at elevated temperatures can be explained on the basis of a disruption of cellular membranes. They found that supplementation of the culture medium with lecithins of cephalins permitted growth to occur at temperatures that were otherwise lethal. In a series of studies on the effects of hyperthermia on phosphorous metabolism [5, 6], it was found that 2-aminoethyl phosphonic acid was released when cultures of *Tetrahymena*

were incubated at 34°C. This compound, which has been identified as a component of cellular membranes in this ciliate [29], is already accumulating 30 min after cells are transferred to 34°C [5]. Thus, it seems that one of the earliest effects of hyperthermia is to disrupt phospholipid-containing membranes within the cell.

It has been suggested [1, 7] that the lesions that accompany metabolic stresses such as anoxia, starvation, and exposure to various chemicals may result from a disruption of the lysosomal membrane with subsequent release of hydrolytic enzymes into the cell sap. However, it appears in many cases that solubilization of lysosomal enzymes, if it does occur at all, follows the cell damage, and is itself a secondary effect [8, 30]. The present results also support this view. Neither acid phosphatase nor cathepsin activity was solubilized by prolonged temperature shock. After 24 h, when cells were near death, the percentage of enzyme in the particulate fraction was as great as or greater than that of log phase cells. In fact, the ultrastructural studies indicate that there is an increase in the number and the contents of autophagic vacuoles during the course of the temperature shock. Figs 4, 5, and 12 show that considerable membranous material accumulates in such vacuoles. This material is presumably derived from disrupted cell organelles. However, our results do not rule out the possibility that some of the lesions occurring in heat-shocked cells might result from a swelling of lysosomal membranes, with a release of toxic breakdown products into the cell sap. Thus, studies by Eichel [9, 10] suggest that the extreme lability of oxidative phosphorylation in homogenates of *Tetrahymena* is due to inhibition by lysolecithins which are released from cell particulates. Phospholipase has recently been found in lysosome-like particles [13, 32].

Our ultrastructural studies reveal that hyperthermia produces severe damage to various cell organelles. The effects on mitochondria are quite conspicuous. A loss of tubules from these organelles is apparent in the early stages of heat shock and after 11 h, many mitochondria are

virtually devoid of tubules. Interestingly, many of the tubules were found in autophagic vacuoles. It has been suggested [25, 26] that hyperthermia has an inhibitory effect on oxidative phosphorylation in *Tetrahymena*. While the work is not unequivocal (studies were conducted at normal temperatures on homogenates of cells which had been exposed to short temperature shocks), our results would provide an ultrastructural basis for the interference with normal mitochondrial function.

Of further interest is the observed swelling of cellular organelles which are tentatively identified as peroxisomes or primary lysosomes. These are quite swollen, even after temperature shocks of 30–60 min, often becoming much larger in diameter than mitochondria. Although the possibility exists that the swelling is an artifact of fixation, this appearance has not been observed in normal or starved cells [22] and is consistently observed in heat-shocked cells. These bodies seem to be reduced in number in cells exposed to elevated temperature for 11 h. Preliminary studies indicate that the specific activities of both mitochondrial and peroxisomal enzymes decrease sharply when *Tetrahymena* is incubated at 33.5°C for prolonged periods.

Abnormalities in nuclear structure have been reported in light-microscopial studies of cells maintained at supraoptimal temperatures [4, 17]. In our studies, large bodies, such as those shown in figs 6 and 7 were quite common in cells subjected to prolonged temperature shock. Large nucleolar fusion bodies have been reported in cells subjected to cyclic heat shock [4, 12]. The possibility exists that the bodies shown here result from a continuation of the fusion process. However, the exact nature of these bodies will have to await further study, especially since they were found in the cytoplasm, as well as in the nucleus. (The transfer of nucleolar-like bodies from nucleus to cytoplasm has been observed in this organism [4, 12, 22].)

Another aspect of the present study is the virtual absence of RER in temperature-shocked cells. The RER is usually quite extensive in normal cells, being found in association with

many of the mitochondria (fig. 3), as well as below the cell membrane. In cells incubated at 33.5°C for 11 h, it has all but disappeared. The disruption of the RER could account for the cessation of protein synthesis that was observed.

The results observed here seem to be consistent with the suggestion [28] that the lesions observed in *Tetrahymena* that have been subjected to hyperthermic conditions result from disruption of or interference with the normal functioning of phospholipid-containing membranes. The disruption of mitochondria and the ER are consistent with this view, as are the swelling of peroxisomes or primary lysosomes, the presence of numerous membranes within autophagic vacuoles, and the general appearance of the cells after prolonged exposure to elevated temperature. Biochemical studies to corroborate this view are currently in progress.

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