SYNTHESIS OF CORTICAL PROTEINS IN
TETRAHYMENA

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SUMMARY

The synthesis of proteins of the oral apparatus in the ciliate Tetrahymena pyriformis has been studied in relation to the development of this organelle system within the cell. Oral proteins were labelled in both logarithmic phase and synchronized cells by growing the cells in the presence of tritiated amino acids. Estimates of the relative amounts of radioactive protein within the old and developing oral structures under various experimental conditions were made by radioautography of the structures after isolation from the cells. Evidence is presented that (i) mature oral structures undergo constant protein turnover; (ii) new oral organelle systems are constructed largely from proteins which are made prior to organelle differentiation and which are contained within a cytoplasmic pool and possibly also in pre-existing oral apparatuses; (iii) there are regular features in the label incorporation and conservation data which correlate well with morphological changes known to occur in the anterior (old) oral apparatus just prior to division; (iv) incorporation of labelled amino acids into the protein of oral structures does not occur during the synchronizing heat shocks; and (v) incorporation of label into the developing oral primordium does occur during primordium development, although it is not known whether these structural proteins are those demonstrated by other studies for which synthesis during development is an absolute requirement.

INTRODUCTION

The nature of a given cell and the functions it performs are determined to some extent by the subcellular structures it has or is capable of forming. Therefore, the problem of the mechanisms regulating organelle formation is of basic importance in the related areas of cell function, cell development, and cell heredity. One of the fundamental questions which has been recognized recently in this area is the relation of protein synthesis to organelle development. A simple and universally applicable answer to the question has not appeared. It has been shown, in recent studies of flagella formation in bacteria (Martinez & Gordee, 1966; Suzuki & Iino, 1966), that flagellin monomer is present within certain bacterial cells and that formation of flagella can take place making use of these precursor proteins in the absence of further protein synthesis. A similar situation was found for eucaryotic flagella by Auclair & Siegel (1966), who studied the regeneration of cilia (flagella) in sea-urchin embryos. On the other hand, a requirement for de novo synthesis of protein during regeneration of flagella in protozoan flagellates has been demonstrated (Rosenbaum & Child, 1967).
The present study is concerned with elucidating relations which may exist between protein synthesis and formation of the feeding organelle system ('oral apparatus') in the eucaryotic protist, *Tetrahymena pyriformis*.

The oral apparatus (OA) of *Tetrahymena* is situated near the anterior end of the cell. It consists primarily of an interrelated system of cilia, basal bodies, and intracellular fibrillar material associated with a cavity in the cell surface which is used for feeding. During cell division, the oral apparatus at the anterior end of the cell is retained by the anterior daughter, while the posterior daughter receives a newly formed oral apparatus which arises in the mid-equatorial region of the parent cell. The new oral apparatus first appears as a primordium consisting of an unorganized field of basal bodies. These later organize into the four groups characteristic of the mature OA (see Figs. 11-13), cilia grow out from most of them, and intracellular fibrous connexions are formed. The oral cavity is formed late in development (see Frankel, 1962, and Williams & Zeuthen, 1966, for more complete descriptions of the course of oral development).

The OA of *Tetrahymena* is in certain fundamental respects similar to the metazoan mitotic apparatus (MA). It consists of basal bodies ('centrioles') and associated fibrous material which is predominantly microtubules. In addition, the OA, like the MA, is formed anew during each cell division.

In synchronized cells, development of the OA is phased in the population and proceeds in association with the synchronous cell division. This synchronous organelle development has been studied from a number of points of view, including its relation to the synchronizing treatment and the synchronous division (Williams, 1964a, b; Williams & Zeuthen, 1966). The relation of protein synthesis to OA development has been studied indirectly by the use of inhibitors of protein synthesis (Frankel, 1962, 1967). The data show that *de novo* synthesis of proteins is apparently an absolute requirement for oral organelle development in *Tetrahymena*, as it is for MA formation in sea-urchin eggs. The present study of protein synthesis in relation to OA development is based upon radioautographic estimation of tritiated amino acids incorporated into OA protein in logarithmic and synchronized cells under various experimental conditions. It will be shown that the OA is further like the MA (Wilt, Sakai & Mazia, 1967) in that, the requirement for *de novo* synthesis notwithstanding, it is constructed largely from precursor proteins previously synthesized and stored within the cells.

**MATERIALS AND METHODS**

**Cells, growth and synchronization**

An amicronucleate strain of the ciliate *Tetrahymena pyriformis* GL was used in this study. The growth medium used routinely was 2 % proteose-peptone with 0.4 % liver fraction L and salts as described previously (Plesner, Rasmussen & Zeuthen, 1964). Stocks of *Tetrahymena* were grown at 28°C in 7 ml of the standard medium in screw-cap tubes and transferred every other day. These stocks were used to inoculate the larger flask cultures used in the experiments. For the experiments, cells were grown in either 60 ml of medium contained in 500-ml Erlenmeyer flasks or in 30 ml of medium in 250-ml flasks. In some experiments it was found that better lysis of the cells for OA recovery could be obtained in a dilute medium;
the standard medium, diluted to 20%, was employed. In other experiments, the cells were washed and suspended in a non-nutrient medium. For this, inorganic medium (IM) used by many workers for Tetrahymena was employed (Plesner et al. 1964). In all experiments involving synchronization, cells were grown in 60 ml of stock culture for between 10 and 12 h before the beginning of the synchronizing treatment. Synchrony was induced by application of the standard seven-shock heat treatment (Zeuthen, 1964) administered by automatic synchronizing devices described previously (Williams, 1964a, b; Plesner et al. 1964). The heat shocks (34°C) were of 30 min duration, interspersed with 30-min periods of return to the optimum temperature (28°C). The time when the last heat shock ends is designated EH.

The time of the 'division maximum' was always estimated in an experiment, this being the time point at which the greatest number of cells cleaving are in some visible stage of this process. In general, division maxima from 85 to 90% were observed between 65 and 75 min after EH in the experiments. Cell growth was followed with a Coulter counter in the experiments with unsynchronized cells.

**Oral apparatus isolation**

The oral apparatus and various stages in the development of the oral primordium were isolated and studied radioautographically in the present study. The isolation method used was that previously described by Williams & Zeuthen (1966). Briefly, the cells were concentrated by centrifugation for 3 min at 2000 g in 40-ml conical centrifuge tubes. After discarding the supernatant, 12 ml of 1.5 M tertiary butyl alcohol was added to the cell pellet and mixed by hand or in a vortex mixer. The lysate was then transferred to round-bottomed culture tubes for collecting the OA fraction by differential centrifugation. After adding an equal volume of distilled water to the lysate, the suspension was centrifuged for 10 min at 2000 g. The supernatant was then discarded and the OA fraction in the pellet taken up in a small amount of water and spotted on slides as described below.

**Labelling and radioautography**

Cellular protein was tritium-labelled by incubating the cells with either tritiated leucine, histidine, or a mixture of histidine and phenylalanine. [4,5-3H]L-leucine (specific activity 5 c/mm) was obtained from New England Nuclear. [3H]L-histidine (specific activity 5 c/mm), L-phenylalanine (specific activity 27.5 µc/ml) and L-histidine (specific activity 25 µc/ml) were obtained from Schwarz. Concentrated solutions of radioactive amino acids were prepared, then small quantities (between 70 and 600 µl, depending on the experiment) were added to the experimental cultures to achieve the final concentrations of amino acids desired. When removing radioactive amino acids from the cultures, the following procedure was used. The culture was plunged into a bucket of water containing ice and swirled intermittently for 3 min. The cells were then collected in sterile, pre-cooled conical centrifuge tubes (40 ml) by centrifuging at 5 °C for 2 min at low speed. The supernatant can be decanted from such cooled cultures with very little loss of cellular material. After discarding the supernatant, the cells were suspended in an equivalent volume of sterile wash medium (inorganic medium or dilute PPL medium). The cells were again collected and the second supernatant discarded. The cells were then taken up in the desired volume of medium with or without unlabelled amino acids added, depending on the experiment. Synchronized cells were washed after the fifth heat shock, then returned to 28 °C, after which two additional heat shocks were administered in order to restore good synchrony. In one experiment, unlabelled amino acids were added at EH. In experiments dealing with unsynchronized cells, unlabelled amino acids were added to the washing medium and kept in the culture subsequently. L-Leucine, L-histidine, and L-phenylalanine were used in the chase experiments.

Relative levels of radioactivity in OA proteins under various conditions were estimated radioautographically. The oral organelles, isolated and concentrated as described above, were taken up in a small amount of distilled water with capillary pipettes and placed in small spots aligned in rows on clean microscope slides. These were dried under a light stream of warm air from an electric dryer. The slides were then fixed for 10 min in acetic acid-ethanol, rinsed in...
95% alcohol, and extracted for 15 min in cold trichloroacetic acid. They were then rinsed in 80% alcohol, dehydrated and air-dried. The slides were coated with either Kodak NTB-2 or Ilford E-5 liquid emulsion. The coated slides were stored in the dark in the presence of Drierite prior to developing. Unless otherwise stated, slides from an experiment were developed after 21 days. Slides were developed for 2 min in Dektol developer, rinsed in tap water, fixed for 3 min in Eastman F-5, and rinsed for 20 min in running tap water. They were finally rinsed in distilled water and air-dried. The best procedure for counting grains in the developed radioautograms was found to be simply adding a drop of water and a coverslip to the slide, then observing the wet-mount with phase optics. A sample size of 60 OA was used in most cases for determining the average number of grains per OA for each point in the experiments. Where the sample size was larger or smaller, this is stated in the text. In general, counts from developing primordia are based on smaller sample sizes because they were more difficult to recover.

RESULTS

Incorporation studies

The general condition of the isolated OA is indicated in Fig. 13. Figures 11 and 12 are isolated oral primordia at two different stages in progressive differentiation. The completed OA and all stages in primordium development have been isolated and studied radioautographically. Figure 14 shows the distribution of grains in the radioautogram obtained from the OA in Fig. 13.

In experiments of this kind, it is important to determine whether significant amounts of radioactive proteins can be adsorbed by the OA during the isolation procedure. If so, the radioautographic estimates of activity will not reflect incorporation of precursors into the OA as integral elements of structure. For this reason, an experiment was...
designed to test for adsorption. A population of cells was grown and divided into two flasks containing 20 ml of culture each at the same cell density. Cells in one flask were lysed immediately and the non-radioactive OA fraction was isolated. The cells in the other flask were incubated through 4 cycles of the heat-shock synchronizing treatment with tritiated histidine at a concentration of 10 \( \mu \text{c/ml} \). These cells were subjected to a synchronizing treatment in order to permit protein synthesis while preventing division, thus keeping the number of OA to be recovered from this sample approximately the same as in the unlabelled population. At EH plus 15 min, the radioactive cells were lysed with an aliquot of butyl alcohol to which had been added the unlabelled OA fraction from the initial flask. The procedure for lysis involves vigorous mixing, and therefore should provide optimum conditions for adsorption of proteins in the brei by the non-radioactive OA in the mixture. Subsequently, the pooled OA fraction was isolated and washed by differential centrifugation. The oral organelles were then prepared for radioautography and grain counts were made in order to see whether the unlabelled OA material showed radioactivity as well as the OA from labelled cells. A total of 200 OA were counted in this experiment. The results are presented in the histogram in Fig. 1. It is apparent that adsorption of some radioactive protein did occur under the conditions of the experiment, but that the amount of this was small compared to the level of radioactivity in the OA population from labelled cells. The two populations in the total OA fraction are clearly separate in the histogram.

The next experiment was designed to give information about the time of synthesis of proteins incorporated into developing oral structures in synchronized cells. Two identical cultures were synchronized by the application of 4 heat shocks. In one culture equal quantities of tritiated histidine and phenylalanine were added to a final level of 2.5 \( \mu \text{c/ml} \) at the end of the synchronizing treatment ("post-labelled"), and in the other culture the same concentration of labelled amino acids was added just prior to the beginning of the synchronizing treatment ("pre-labelled"). The expectation was that if oral development following EH makes use of proteins synthesized exclusively during this period, the OA isolated at division from the post-labelled cells should contain nearly as much radioactivity as those in the pre-labelled cells. On the other hand, if oral development after EH makes exclusive use of proteins synthesized before the end of the synchronizing treatment, oral apparatuses isolated at division from the pre-labelled cells should contain significantly more radioactivity than those isolated from the post-labelled cells.

The results, presented in Table 1, do not confirm either hypothesis adequately. The newly formed oral apparatuses, isolated at EH plus 65 min, contained significant amounts of radioactive protein in the post-labelled cells, but the amount is significantly lower than the amount found in the pre-labelled cells. These data are best explained by the idea that the development of the oral apparatus in synchronized cells involves proteins which are synthesized both during and after the synchronizing treatment. An unexpected finding in this experiment was that the old anterior oral apparatus, which, unlike the new oral apparatus, is presumably undergoing no development, also incorporated significant amounts of radioactive amino acids in both the pre-labelled and post-labelled cells (Table 1).
The relative rates of incorporation into the old oral organelles and into the developing primordia were determined during the last synchronizing heat shock and at subsequent intervals until the synchronous division. A 60-ml culture of logarithmic phase cells was synchronized by a series of 5 standard heat shocks. After the fifth shock the culture was washed once and the cells taken up in 50 ml of the dilute medium. Five ml of this cell suspension were removed to each of 10 50-ml flasks. All 10 flasks received 2 more synchronizing heat shocks. At some specific time for each flask except one, tritiated histidine was added to a final concentration of 14 μC/ml and incubated with the cells for a 10-min interval. The cells in each flask were lysed at the end of this interval and the OA fractions isolated and prepared for radioautography. The cells in the tenth flask were not treated but were used to observe the time and extent of

Table 1. Incorporation of labelled amino acids into the oral structures of Tetrahymena

(In one culture (‘post-labelled’) equal quantities of tritiated histidine and phenylalanine were added to a final concentration of 25 μC/ml at the end of the synchronizing treatment. In the other (‘pre-labelled’), the same concentration of labelled amino acids was added at the beginning of the synchronizing treatment. Oral structures were isolated at the times indicated and relative amounts of radioactivity in oral proteins were estimated radioautographically (grain counts). Sixty oral apparatuses (OA) of each type indicated were counted in each sample. See text for further details.)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Time of isolation</th>
<th>EH plus 40 min</th>
<th></th>
<th>EH plus 65 min</th>
<th></th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>New OA</td>
<td>Old OA</td>
<td>New OA</td>
<td>Old OA</td>
</tr>
<tr>
<td>Pre-labelled</td>
<td>—</td>
<td>20.5 ± 0.87</td>
<td>35.8 ± 0.73</td>
<td>30.2 ± 0.89</td>
<td></td>
</tr>
<tr>
<td>Post-labelled</td>
<td>—</td>
<td>10.5 ± 0.71</td>
<td>25.3 ± 0.64</td>
<td>18.9 ± 0.64</td>
<td></td>
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</table>

The observed incorporation rate changes in both the anterior, morphostatic OA, and in the developing primordium are indicated in Fig. 2. It appears that incorporation did not occur during the heat shock, when account is taken of the level which is due to adsorption (Fig. 1). Following EH plus 15 min, incorporation occurs in both old and new oral structures with increasing rate until just before the synchronous division, at which time a decline in rate begins. A probit analysis of the grain-count distributions obtained in this experiment is presented in Fig. 3. All distributions were
Fig. 2. Relative rates of incorporation of labelled amino acid into protein in the anterior (old) oral apparatus and the developing oral primordium during and after the last synchronizing heat shock. Each point represents the average number of grains in radioautographs of a sample of OA isolated from cells incubated for a 10-min interval with 14 μg/ml of tritiated histidine just before isolation. Zero time marks the end of the heat shock (34 °C), after which the cells were returned to the optimum temperature (28 °C) and kept until the synchronous division occurred (arrow). The brackets indicate the standard error of the mean in each case. Sample characteristics are further described in the legend to Fig. 3. See text for discussion.

Fig. 3. Probit analysis of grain-count distributions from which means in Fig. 2 were calculated. The distribution of grains in primordia (●) and in anterior OA (○) at the times indicated are plotted. Straight lines (fitted by eye) suggest log-normal (monomodal) distributions of grains in most cases. Bimodality is suggested for the primordia at 55 min and for the anterior OA at 45 and 55 min after EH. From left to right the ranges and sample sizes (N) are as follows. Primordia at 25 min: 4-20, N = 13; 35 min: 11-22, N = 12; 45 min: 15-36, N = 17; 55 min: 13-45, N = 25; 65 min: 9-29, N = 39; 75 min: 11-32, N = 50. Anterior OA at -10 min: 0-15, N = 63; 15 min: 0-11, N = 60; 25 min: 3-16, N = 60; 35 min: 5-10, N = 60; 45 min: 4-29, N = 61; 55 min: 4-33, N = 60; 65 min: 4-34, N = 62; 75 min: 5-24, N = 60.
monomodal except those for oral structures determined just prior to the observed declines in incorporation rate. Bimodality at these times probably reflects heterogeneity in the population with respect to the rate change which occurs (see Discussion).

Conservation studies in logarithmic cells

The studies described in this section are 'pulse-chase' experiments, in which the OA proteins were labelled and then the level of radioactivity in the old and newly formed structures followed over a period of time after withdrawal of the radioactive precursors. In the first experiment, the oral organelles were labelled by incubating a 30-ml cell culture in dilute medium with 10 μg/ml of tritiated histidine for 12 h at

28 °C. At the end of this interval the cells were washed twice with fresh dilute medium and then suspended in 20 ml of dilute medium containing unlabelled histidine at a concentration 100 times the concentration of labelled histidine used to label the cells. This was immediately used to inoculate a litre flask containing 125 ml of dilute medium with unlabelled histidine at 100 times the concentration of labelled histidine. The flask was returned to 28 °C and incubated with gentle shaking. The subsequent
growth was followed by periodic counting of samples with a Coulter counter. The level of radioactivity in the oral organelles was determined initially and at intervals following this by isolation and radioautography of the OA fractions from 10-ml samples. Each determination is based on grain counts of 80 individual OA per sample. In this experiment the radioautography slides bearing oral organelles were incubated for 14 days before developing. The results of this experiment are presented in Figs. 4–6.

It will be seen in Fig. 4 that there was a 2-h lag period before logarithmic growth began. This is probably due to the thermal and mechanical shocks involved in preparing the radioactive cells for inoculation into non-radioactive medium. During the lag there was a slight loss in radioactivity from oral proteins. The cells then grew logarithmically for 3 doublings of population size, with a generation time of 3·0 h. After 3 doublings the experiment was terminated. By frequent determination of cell number it was possible to isolate OA fractions at the end of the lag phase and at the times that 1, 2 and 3 doublings of cell number had occurred. It can be seen that there
was a steady decline in the average amount of label in the OA with time during logarithmic growth and that this decline in 'specific activity' is not log-linear.

Inspection of histograms prepared from these data (Fig. 5) reveals that the decline in mean radioactivity in the OA was not due to conservation of label in the pre-labelled OA and a dilution of this level of radioactivity by formation, during growth, of new organelles made from non-radioactive proteins. Each frequency distribution from which the mean was calculated appeared to be monomodal, with no evidence of two separate classes of OA based upon radioactive protein content. This surprising result prompted a statistical test for bimodality using the probit analysis (Fig. 6). The high resolution of this test revealed a slight tendency toward bimodality at the beginning of logarithmic growth and at the first doubling, but not after that. This bimodality during early growth is probably due to handling of the cells. The complete lack of bimodality at the second and third doublings indicates that pre-labelled OA and those formed long after the chase are not different in their content of labelled protein. This, in turn, is interpreted to mean (i) that there is a progressive loss of label from the pre-labelled OA, and (ii) that new structures, formed long after the chase, are assembled from radioactive precursors. The source of this radioactivity will be considered in the Discussion.

The experiment was repeated under identical conditions with identical results.
Some differences were observed, however, in an experiment under slightly altered conditions. In this experiment cells were grown in 10 ml of 2% proteose peptone and labelled by incubating for 30 min with a mixture of tritiated phenylalanine at 27.5 μCi/ml and tritiated histidine at 25 μCi/ml. The cells were then washed twice (at room temperature) in 2% proteose peptone and suspended in this medium with L-phenylalanine and L-histidine at a concentration of 1 mM each. Growth was followed by cell counts (manually) until one doubling had occurred. The radioactivity in OA protein was determined. The results are presented in Fig. 7. The growth pattern was about the same as in the previous experiment, including a 2-h lag phase, whereas different initial levels of radioactive protein in the OA material are seen in the two experiments.

Fig. 7. Conservation of labelled protein in oral structures during lag and logarithmic phases of growth following removal of radioactive amino acids and addition of an excess of unlabelled amino acids. Oral protein was labelled by incubating cells in 2% proteose-peptone with a mixture of tritiated phenylalanine (27.5 μCi/ml) and tritiated histidine (25 μCi/ml) for 30 min at 28°C. The cells were washed and suspended in fresh medium with L-phenylalanine and L-histidine at 1 mM each. Growth was followed through one doubling by periodic counting in a Sedgwick-Rafter cell (●). The relative amounts of radioactive protein in oral structures were determined periodically (○) by isolation and radioautography. Further sample characteristics are presented in Fig. 8. Comparison with Fig. 4 shows a more rapid loss of label from the OA during the lag phase.

This may in part reflect differences in incorporation time and in the type and concentration of labelled amino acids used. In this experiment there was a more rapid loss of label from the OA during the lag phase. The reason for this difference is not understood. Probit analysis of the grain-count frequency distributions in the present experiment revealed that the distributions at all times were monomodal (Fig. 8), again indicating that the pre-labelled OA lost label and that new ones were formed from labelled precursors.
Log. grains (1 unit=doubling)

Fig. 8. Probit analysis of the grain-count distribution from which means in Fig. 7 were calculated (times indicated). The curves (fitted by eye) suggest log. normal (monomodal) distributions at all times. In each case 80% of the counted structures are between the horizontal lines. The monomodal distributions indicate that OA formed in the presence of labelled amino acids cannot be distinguished from those formed after the chase. From left to right the ranges and sample sizes (N) are as follows: 65 min: 0-33, N = 84; 90 min: 1-29, N = 92; 105 min: 0-18, N = 134; 175 min: 0-14, N = 101; 260 min: 0-17, N = 103.

Conservation of labelled proteins in synchronized cells

The conservation of labelled protein in oral structures was also studied in synchronized cells. Cells were grown for 10 h in 60 ml of the standard growth medium and then subjected to the synchronizing treatment. Tritiated leucine (10 μc/ml) was added to the culture just prior to the first heat shock in the series. After the fifth heat shock the cells were cooled, washed twice to remove the labelled amino acid, and suspended in inorganic medium. They were then returned to 28 °C and subsequently given two additional heat shocks to ensure good synchrony. Immediately after the last heat shock, unlabelled leucine was added to the culture at a concentration 1500 times that of labelled leucine used. Five-ml samples were removed at intervals after this and prepared for radioautographic estimation of labelled protein in the oral organelles as in the previous experiments. The results are presented in Figs. 9 and 10. Separate data were obtained for the anterior OA and the developing primordium at EH plus 55 min and before this. Unlike the situation in the incorporation-rate experiment (Fig. 2), new and old oral structures could not readily be distinguished from each other after this time. This is due in part to the earlier division which occurred in the present experiment. Estimates of radioactive protein in all oral structures after EH plus 55 min and in the anterior OA prior to this time are based on grain counts in samples consisting of 60 OA per sample. Smaller samples were used to estimate radioactivity in the developing primordium because these were much more difficult to recover (see Fig. 10).

The results (Fig. 9) show that both the oral primordium and the anterior oral apparatus increased in radioactive protein content until approximately EH plus 55
min, with the rate of increase being more rapid in the primordium. As in the previous experiments with logarithmic cells, this indicates that the oral organelles continue to incorporate radioactive precursors into structure after radioactive amino acids have been washed out and chased with a large excess of unlabelled amino acids. The level

Fig. 9. Conservation of labelled protein in oral structures in synchronized cells. The cells were exposed to tritiated leucine \((10 \mu\text{c/ml})\) in standard growth medium from the beginning of the synchronizing treatment until the end of the 5th heat shock. The label was removed by washing and the cells were suspended in inorganic medium. Two more heat shocks were given to ensure synchrony. After the last heat shock, unlabelled leucine was added at a concentration 1500 times that of labelled leucine used. Samples were removed at intervals, OA were isolated, and radioactive protein content was estimated radioautographically. Radioactivity was determined separately for the old oral apparatus (O—O) and the developing primordium (● — ●) prior to the first synchronous division. Following this, all OA recovered were mature structures. The times of the first and second divisions are indicated by arrows. The brackets indicate the standard error of the mean in each case. Other sample characteristics are given in the legend to Fig. 10.

of radioactive protein declined just prior to the first synchronous division and remained essentially at a constant level until the end of the experiment, which was considerably after the second synchronous division. In this experiment the first division maximum was 90% at EH plus 65 min and the second division maximum (visually estimated) occurred at approximately 150 min after EH. The grain-count frequency distributions from each determination were studied by probit analysis to test for bimodality. It can be seen in Fig. 10 that nearly all distributions were monomodal, indicating no difference in labelled protein content between fully formed OA which were pre-labelled and those which completed development or were formed entirely after the chase. This is consistent with the data from logarithmic phase cells. The one distribution which shows a slight tendency toward bimodality was obtained from a mature OA population isolated from cells shortly before the first synchronous division, i.e.
at EH plus 45 min in this experiment (Fig. 10). This will be discussed below in relation to morphological changes which are known to occur in the anterior oral apparatus just prior to cell division.

DISCUSSION

Microscopic study of the isolated oral apparatus (Nilsson & Williams, 1966; Williams & Zeuthen, 1966) has not yet included high-resolution electron microscopy, so detailed information on the structural elements present is lacking. The information available, however, together with a knowledge of oral apparatus ultrastructure in Tetrahymena as determined from whole cells (Nilsson & Williams, 1966; Williams & Luft, 1968), permits a gross characterization. It has been established that the isolated oral apparatus is devoid of cilia, therefore the present study does not bear directly on the question of ciliogenesis. Aside from cilia, the OA in vivo consists of pellicular material, many basal bodies (approximately 170 per OA), abundant quantities of microtubular fibres, and a lesser amount of fine filamentous material. The descriptions of the isolated OA available suggest that it consists predominantly of basal bodies and associated microtubules. Therefore the present incorporation studies probably deal primarily with the synthesis and assembly of the proteins of these structures. However, further study will be required to determine with certainty whether proteins
Synthesis of cortical proteins in *Tetrahymena*

of one or both elements are involved in the study, and whether proteins of other elements may also be represented by the data.

The adsorption experiment described (p. 147) shows that only a relatively small amount of non-OA protein is adsorbed to the structures during isolation: therefore the radioactive protein determinations using the isolated OA do reflect incorporation of labelled amino acids into proteins of some or all of the structural elements present.

The anterior end of each cell possesses a formed oral apparatus which is far removed from the site of primordium development. With the exception of a brief period just prior to division (discussed below), this OA may be considered to be morphostatic, i.e. it undergoes no visible morphological changes. Nevertheless, it was found that labelled amino acids are extensively incorporated into anterior OA protein, and further, that label incorporated into protein is continuously lost from this structure in chase experiments. This suggests that the anterior OA, which is morphologically stable, undergoes protein turnover continuously. The loss of label from mature OA structures could be due either to a migration of labelled protein from the structures, or a degradation of these proteins (discussed below).

Previous work with inhibitors of protein synthesis has indicated that there is a requirement for de novo synthesis during oral morphogenesis in *Tetrahymena* (Frankel, 1962, 1967). Development of the oral primordium in strain GL is stopped, either with or without complete resorption of the partially formed structures, if protein synthesis is blocked at any point prior to a time just preceding the onset of cytoplasmic cleavage. After this time, continued primordium development will occur in the absence of protein synthesis. The present study shows that proteins which are synthesized during development may become incorporated into the developing primordium. These proteins may represent the de novo synthesis requirement indicated by the inhibitor studies. Another possibility, however, is that the proteins synthesized and incorporated into OA structure while development is occurring may not be those for which synthesis during development is an absolute requirement. They may instead be simply more of the type previously synthesized and stored by the cell (see below). They would be used under the conditions of the experiment but not required, whereas protein represented by the de novo requirement could play some functional role in promoting assembly without itself entering into OA structure. Additional information is needed in order to distinguish between these alternatives.

The experiment presented in Fig. 9 shows that incorporation of labelled amino acids into OA proteins occurs during the synchronizing treatment. However, this incorporation must occur during the intershock intervals, because it is demonstrated in Fig. 2 that no incorporation occurs during the heat shocks. Here again more than one interpretation is possible. The synthesis of building blocks or of a catalytic element may be affected, or the disturbance by heat may be mostly or only at the level of assembly processes. For further discussion of this problem, see Zeuthen & Williams (1969).

Although both the anterior OA and the developing oral primordium incorporated amino acids into organellar protein to significant degrees between EH and the synchronous division, the rate of incorporation was much greater in the primordium
following EH plus 25 min (Fig. 2). Incorporation of amino acids into oral proteins has been reported for *Stentor* by de Terra (1966). She also noted that incorporation was greater in morphogenetic (regenerating) structures than in morphostatic ones. In *Tetrahymena* the rate of incorporation in both oral systems within the cell increased until just before cleavage. At this time the rates declined. The decline in the anterior OA may be related to morphological changes known to occur. At this time the oral apparatus undergoes a structural 'simplification' in which the deep fibre is resorbed, the buccal cavity is lost, and the remaining structures lie on the surface of the cell (Frankel, 1962; Williams & Zeuthen, 1966). This implies a net loss of protein within the OA, and this change may be reflected in a reduced rate of incorporation. In addition, it has been shown that food vacuole formation is impaired at the time these structural changes are occurring (Nachtwey & Dickinson, 1967). Therefore, the reduced incorporation rate may be at least in part due to a reduced rate of uptake of labelled amino acids into the cells. The probit analysis of the frequency distributions of grain counts in samples of anterior OA in the incorporation rate experiment show a slight tendency toward bimodality at EH plus 45 and 55 min which is not seen otherwise. These times coincide approximately with the onset of the structural changes and the related impairment of food-vacuole formation. If these changes occur at about the same time in all cells, it is likely that some cells in the population will have initiated them before others, due to imperfect synchrony. This would explain the temporary appearance of two classes of oral structures on the basis of radioactive protein content.

The same sort of bimodality in the anterior OA population appears at EH plus 45 min in the chase experiment (Fig. 10). Because the cells were labelled at a previous time in this experiment, the bimodality cannot be due to a change in the rate of uptake of amino acids. It must, in this case, be directly related to the structural changes which occur in the anterior OA at this time.

The developing oral primordium, in addition to the anterior OA, undergoes a decline in incorporation rate just prior to division. There is also the temporary appearance of bimodality in the distribution of grain counts in the primordium population at the beginning of this decline in incorporation rate. These data could be explained on the basis of a reduced rate of amino acid uptake by the cells, as discussed above. On the other hand, it is surprising that the anterior OA and the primordium, which are very different in their radioactive protein content at EH plus 55 min in the chase experiment (Fig. 9), are indistinguishable on the basis of probit analysis of radioactive protein content just 10 min later. Perhaps significantly, they also become morphologically similar at this time. These data suggest the operation of some external, pervasive regulatory influence which brings the developing primordium and the anterior OA into step with each other late in the cell cycle. This is similar to a situation found in *Stentor* by Tartar (1966). He found that two regenerating oral primordia which are initially in different stages, produced in a single cell by microsurgical grafting methods, have a strong tendency to synchronize their development and complete the later stages of oral differentiation together.

The chase experiments with both logarithmic and synchronized cells were designed
to determine patterns of conservation of labelled proteins within the oral apparatus during growth and division. The data show that proteins are steadily lost from pre-labelled structures in logarithmic cells whereas the loss is not as apparent in synchronized cells, at least until after the second synchronous division. More significantly, perhaps, the data also strongly suggest that proteins used in the construction of oral organelles may be synthesized within the cells and stored in a precursor pool, for use over a long period of time. The curves indicating the decline in 'specific activity' of the OA after the chase in Figs. 4 and 7 are similar to what would be expected if the OA that were labelled before the radioactive amino acids were washed out and chased with large excesses of unlabelled amino acids, retained their labelled protein, while new OA, formed after this and containing no labelled protein, would progressively dilute the count with time. The histogram in Fig. 5 and the probit analyses of the grain-count distributions obtained at various times after the chase (Figs. 6, 8), however, clearly show that separate populations based upon radioactive protein content cannot be distinguished. The one exception is the slight tendency toward bimodality found soon after the chase in one experiment, and this may be an effect of handling the cells. The fact that all OA are heavily labelled after the chase suggests that the new structures are constructed of precursors which have become labelled as a consequence of the previous exposure to tritiated amino acids.

The possibility must be considered that these labelled amino acids may not have been removed completely by the washing procedure and that it is these compounds which may be incorporated directly into the OA. This is considered unlikely, however, because of the thoroughness of the washing procedure, the large excess of unlabelled amino acids used, and the length of time involved. For example, in the first experiment with logarithmic cells, 7 out of every 8 OA in the sample taken at the third doubling of cell number were formed after the chase, and these all contained significant amounts of radioactivity. Furthermore, at this time 1 out of every 2 OA was formed approximately 10 h after the chase, indicating access to radioactive precursors long after the chase. However, the best evidence that radioactive amino acids have been effectively removed comes from the experiment with synchronized cells. The experiment was intentionally performed under conditions identical with those used by Crockett, Dunham & Rasmussen (1965) in their experiment dealing with conservation of labelled total cell protein in synchronized Tetrahymena pyriformis GL in inorganic medium. In contrast to the present data on OA protein (both the anterior OA and the primordium gained activity after the chase), they found that there was no increase in radioactivity in total cell protein after the chase. Thus radioactive amino acids are probably not available for protein synthesis after the chase under the conditions used in both experiments. Furthermore, because cell division occurred during the interval studied in their experiment whereas no increase in radioactivity within protein occurred in the culture as a whole, the amount of radioactive protein per cell must decline by half at each division. The radioactive protein content per oral apparatus, on the other hand, remained essentially constant through two division cycles (Fig. 9). It is extremely unlikely, then, that the source of label in protein of oral structures formed after the chase can be labelled amino acids in the amino acid
pool. It must be concluded that compounds containing the radioactivity are sequestered in some way specifically for use in OA development. The simplest way to explain this is to suggest that they are sequestered by being previously synthesized into proteins specific for organelle development.

An important question is whether or not the proteins which leave mature OA structures during protein turnover constitute the source of building blocks for construction of new primordia. It is unlikely that they could constitute the entire source in any event, because there is more than twice the total amount of radioactive protein in oral structures after three doublings of cell number in the chase experiment shown in Fig. 4 than there was in oral structures at the beginning of logarithmic growth. Therefore, at least some oral precursor proteins are probably localized in a cytoplasmic pool. On the other hand, the protein which leaves mature OA could conceivably remain intact and re-enter mature OA or enter developing primordia. Degradation of protein to amino acids cannot be demonstrated in growing cells (Crockett, Dunham & Rasmussen, 1965), therefore it seems most likely that the oral proteins which leave mature structures under these conditions do remain intact. Furthermore, the data in Figs. 4-6 suggest that these proteins re-enter a cytoplasmic pool of oral proteins which is continuously supplying proteins to old and new oral structures during protein turnover and development. The indication comes from the fact that after several doublings of cell number in this chase experiment, old OA, which are losing labelled protein, and new OA, which are incorporating labelled protein, cannot be distinguished from each other on the basis of radioactive protein content. This would be expected if old and new structures were exchanging and drawing proteins, respectively, from a common cytoplasmic pool. On the other hand, if the labelled proteins in the old OA were being degraded, this equivalence in radioactivity content after three doublings could only result if the rate of degradation of old OA protein equaled the rate at which new proteins entered the new OA from the cytoplasmic pool over long periods of time. This seems unlikely, and the simplest interpretation would be that proteins from mature OA shuttle back and forth between the cortical structures and a cytoplasmic pool, and it is this pool which is the source of new proteins used in development of new oral primordia. This ‘molecular integration’ of old and developing oral structures is consistent with data presented above which suggest a common regulation of the two structures within a single cell which is seen just prior to cell division. The oral apparatus, then, does not appear to be a static structure. According to the view developed here, its essential characteristic may be that of a morphogenetic field which imparts order to molecular materials which are in a constant state of flux within the system.

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Figs. 11–14. Oral structures isolated from *T. pyriformis* cells by the butanol method employed in the present investigation. Photographed with phase-contrast optics; no stain was used. × 1200.

Fig. 11. Developing oral primordium at the stage in which kinetosomes become grouped as the process of membranelle formation begins. Isolated from synchronized culture in nutrient medium at approximately 50 min after the end of the synchronizing treatment.

Fig. 12. Slightly later stage in the development of the oral primordium, isolated from a synchronized culture in nutrient medium at approximately 60 min after the end of the synchronizing treatment. Note that the three membranelles (upper right in photograph) are well developed.

Fig. 13. Fully formed anterior oral apparatus isolated from logarithmic phase culture. This oral apparatus was prepared for radioautography, but the developed grains do not show because they lie at a different focal level.

Fig. 14. Same oral apparatus as in Fig. 13, but photographed at the level of the developed radioautographic grains. Because the structures and the developed grains lie at different focal levels, no difficulty was experienced in distinguishing grains from structural elements in either the developing oral primordium or the mature oral organelle systems.