# SYNTHESIS OF *PARAMECIUM* SURFACE PROTEINS

## I. Puromycin Insensitive Amino Acid Incorporation

IRVING FINGER, PATRICIA LAVANCHY, and ANN MEANY

From the Department of Biology, Haverford College, Haverford, Pennsylvania 19041

ABSTRACT

The synthesis of a surface protein has been studied in *Paramecium* through double-labeling experiments using [$^{14}$C]- and [$^{3}$H]leucine-labeled bacteria as the source of radioactive amino acid. Over a 4–5 h incubation period, the turnover rate was found to be higher than that of overall cell protein. In addition, the initial label is apparently utilized during the chase period, being incorporated into protein via a puromycin insensitive pathway.



## INTRODUCTION

The surface of *Paramecium* is coated with proteins having a molecular weight of about 300,000, composed of as many as nine subunits (19, 20). An individual cell has the capacity for making at least a dozen kinds of these proteins, but generally at any one time only a single one is found. Through shifts in temperature or any of a number of other environmental factors, one kind of protein can be lost temporarily to be replaced by another (1). Also, different clones of the same genotype differ in their tendencies for maintaining a particular protein (8).

The experiments described here are the first attempts to obtain quantitative data about the events underlying the persistence or transformation of these surface proteins. It appears that the turnover rate is indeed considerably higher than that of the average cell protein and, furthermore, there is evidence that moieties synthesized early in the life of a culture may be assembled later into complete surface protein without passing through a ribosomal pathway. These "precursors" may exist in internal pools or free in the medium.

## MATERIALS AND METHODS

### *Stocks*

Cells of stock 7, syngen 2, of *P. aurelia* were used exclusively. All cultures were maintained in a Cerophyl (Cerophyl Laboratories, Inc., Kansas City, Mo.) medium (4).

### *Purification of Surface Protein*

Preer's (16) method, as adapted for syngen 2 serotypes (6), was used to purify the surface proteins. In this procedure, cells are placed in a saline-alcohol solution and bodies and debris are removed by centrifugation at 10,000 *g* for 10 min. The soluble material is acidified to pH 2 and, after discarding the precipitate formed, the extract is neutralized and brought up to a 35% ammonium sulphate concentration. The soluble fraction is then brought to a final concentration of 60% with saturated ammonium sulphate and the precipitate is recovered. This 35–60% cut, after dialysis overnight against saline, is further fractionated in a 5–25% glycerol gradient by centrifugation in a Beckman Spinco L-2 (40,000 rpm for 10 h using a SW 50.1 rotor, or 35,000 rpm for 20 h with a SW 41 rotor, Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.). The peak of the purified protein appears about 60% of the distance from the meniscus (cf. 18). That this material is chiefly or exclusively surface antigen is indicated by: (*a*) analytical centrifuge experiments (17, 20) which show only one ultraviolet absorbing peak; (*b*) the correspondence between the amount of precipitable antigen in this peak and the absorbing power of this peak (18); and (*c*) the fact that antiserum against this peak yields only one band in gel diffusion.

### *Radioactive Labeling of Protein*

*Escherichia coli* was grown on a minimal-glucose medium with added [$^{14}$C]leucine and then fed to washed paramecia (grown and concentrated from 5 gallon carboys) (5). After 18 h at room temperature in a 2 l Ehrlenmeyer flask, 500 ml of cells were washed and resuspended in fresh culture fluid to which had been added 15 ml of a bacterial suspension containing 35 $\mu$Ci (sp act 5 Ci/mmole) [$^{3}$H]leucine (New England Nuclear Corp., Boston, Mass.) per $6 \times 10^6$ paramecia. Depending on the particular experiment, the contents of a single carboy might be divided among as many as six 500-ml flasks, each with 200 ml of culture fluid. After incubation for an appropriate time, the paramecia were removed by centrifugation and washed with 250 ml of medium. The pellet from this wash was then stored in saline-alcohol at 4°C until purification could be carried out.

Purified surface protein was assayed for $^{14}$C and $^{3}$H label, using a liquid scintillation spectrometer. 0.3 ml of fractions from a glycerol gradient were added to counting vials together with 0.5 ml of methanol and 10 ml of Bray's (2) solution. Two 10-min counts of each sample were averaged and, using appropriate standards, disintegration per minute values were computed.

### *Estimation of Protein*

The method of Lowry et al. (12) was used to determine the protein content of gradient fractions. Usually 0.2 ml aliquots were adequate; for estimates of trichloroacetic acid- (TCA-) precipitable protein 0.05 ml aliquots were used.

### *Nonsurface (TCA Precipitable) Proteins*

To obtain a sample of cell protein lacking surface protein, the bodies and other debris remaining after salt-alcohol extraction were homogenized for 1 min in a Potter-Elvehjem homogenizer. The supernatant of a low-speed centrifugation was then mixed with 50% TCA to yield a 5% solution which was placed at 4°C for 20 min. The pellet from another centrifugation was washed three times with 5.0 ml TCA and then dissolved in 0.5 ml of 0.1 M NaOH (13). Aliquots of this were then removed for protein determination and isotope counting.

To determine whether the TCA-precipitable fraction was relatively free of surface protein initially, the bodies and other debris were homogenized in salt-alcohol and surface protein (i.e., immobilization antigen) was extracted, purified, and assayed in gel diffusion (6). Of the 12 clones examined, eight had no detectible antigen in this fraction. In view of the results described below, even if this fraction was grossly contaminated with surface proteins the interpretation of the data would be unaffected.

## RESULTS

### *Rate of Turnover of Surface Protein*

To follow the loss of old protein and its replacement by new, cells were incubated overnight on bacteria grown on [$^{14}$C]leucine. Once the bacteria had been cleared from the medium (18–22 h), the paramecia were washed and resuspended in fresh culture fluid to which [$^{3}$H]leucine-labeled bacteria had been added. After 4–5 h at room temperature, one subculture was centrifuged, washed, and the antigen was purified by salting-out and density gradient centrifugation. One aliquot was counted and another analyzed for protein. A second subculture was treated in the same way after 8–10 h had elapsed.

Although cells were grown in the same way from experiment to experiment, some as yet unknown variable allowed an increase in total surface protein over the chase period with some cultures and not with others. Nevertheless, the relationships between old and new label are the same. In both kinds of experiments there was generally a considerable increase in the specific activity of the chase ($^{3}$H) label (Table I). Most surprising, however, in half of the clones in which the protein extractable had increased in the 5 or 6 h interval, the specific activity of the original label remained constant. Of the other five clones, three actually showed an increase in specific activity of this label. This is not what would be anticipated for [$^{14}$C]leucine incorporated into a molecule which remained intact or turned over (Table II). Population counts showed that under the conditions of these experiments there was less than a 15% increase in the number of cells.

With prolonged exposure to radioactive bacteria, using a somewhat different procedure, much the same results were obtained (Fig. 1): the old label tended to be conserved and the new label readily incorporated. Whether turnover or net synthesis was responsible for most of the [$^{3}$H]leucine incorporation, to account for the constancy of $^{14}$C labeling one must assume either that this label is recycled (but somehow not diluted with the new [$^{3}$H]leucine), that it is sequestered and does not undergo turnover, or that there exist pools of previously made polypeptides containing [$^{14}$C]leucine.

TABLE I

*Changes in Specific Activities, with Time, of Conserved and Chase-Labeled Surface Protein*

| Experiment | Protein in peak | Time after chase | Initial label ($^{14}C$) sp act | Chase label ($^{3}H$) sp act |
|---|---|---|---|---|
| | *μg* | *h* | | |
| Constant amount of surface protein | | | | |
| 2671-12A | 57 | 6 | 35 | 38 |
| | 51 | 10 | 29 | 76 |
| 2971-12A | 11 | 5 | 44 | 32 |
| | 11 | 10 | 42 | 84 |
| 2471-97 | 25 | 5 | 17 | 19 |
| | 26 | 10 | 18 | 48 |
| 1571-12 | 60 | 4 | 10 | 5 |
| | 60 | 10 | 17 | 62 |
| 1671-36 | 33 | 4 | 18 | 27 |
| | 30 | 10 | 22 | 70 |
| 3471-12 | 36 | 4 | 45 | 26 |
| | 32 | 9 | 35 | 67 |
| 3471-14 | 30 | 4 | 51 | 9 |
| | 30 | 9 | 63 | 54 |
| Increase in amount of surface protein | | | | |
| 1971-97 | 34 | 4 | 22 | 69 |
| | 42 | 10 | 24 | 159 |
| 2471-12 | 66 | 5 | 26 | 40 |
| | 97 | 10 | 22 | 71 |
| 2671-14 | 85 | 6 | 56 | 63 |
| | 113 | 10 | 50 | 196 |
| 1571-22 | 41 | 4 | 44 | 7 |
| | 113 | 10 | 29 | 158 |
| 1671-28 | 72 | 4 | 10 | 10 |
| | 115 | 10 | 12 | 41 |
| 1571-50 | 84 | 4 | 52 | 3 |
| | 123 | 10 | 43 | 37 |
| 3071-20 | 93 | 5 | 28 | 126 |
| | 126 | 10 | 45 | 223 |
| 3071-31 | 39 | 5 | 25 | 126 |
| | 56 | 10 | 44 | 286 |
| 3071-101 | 48 | 5 | 29 | 149 |
| | 60 | 10 | 67 | 381 |
| 3171-14-1 | 15 | 4 | 36 | 14 |
| | 28 | 8 | 30 | 9 |

Specific activity is in terms of disintegration per minute per microgram protein.

TABLE II

*Comparison of Changes in Specific Activities of Surface Protein Observed and Predicted on the Basis of Conservation of Initially Labeled Protein*

| Experiment | % changes in sp act of $^{14}C$-surface protein | |
|---|---|---|
| | % change expected if conserved label is diluted | % change observed in conserved label |
| 1971-97 | −19 | +9 |
| 2471-12 | −32 | −15 |
| 2671-14 | −25 | −11 |
| 1571-22 | −64 | −34 |
| 1671-28 | −38 | +20 |
| 1571-50 | −32 | −17 |
| 3071-20 | −35 | +61 |
| 3071-31 | −44 | +76 |
| 3071-101 | −25 | +131 |
| 3171-14-1 | −87 | −17 |

The percentages are calculated on the basis of data in Table I. Conserved label refers to the initial [$^{14}C$]leucine label. The percentages expected if this isotope was diluted were computed from the change observed in the amount of protein extracted

$$\frac{(t_2 - t_1) \times 100}{t_2}$$

$t_1$ = μg protein made after first time interval.
$t_2$ = μg protein made after total time of experiment.

The fate of the chase $^{3}H$ label in the surface protein was also compared with that of the TCA-precipitable protein (after the extraction of surface protein) (Table III). By one criterion, specific activity, the TCA-precipitable protein turned over or was newly synthesized at several times the rate of the surface protein. However, when the changes in specific activity with time were compared, the surface protein generally appeared to increase much more rapidly than did the other proteins of the cell. Experiments examining the effect of puromycin on $^{14}C$ and $^{3}H$ incorporation were devised to resolve this paradox.

## *Effect of Puromycin*

Perhaps the most obvious explanation for the increase (or for the constancy) in the specific activity of $^{14}C$-labeled surface protein is that all of the [$^{14}C$]leucine derived from the labeled bacteria is not exhausted at the time the chase label is introduced. To determine whether this was so, puromycin, an inhibitor of polypeptide chain

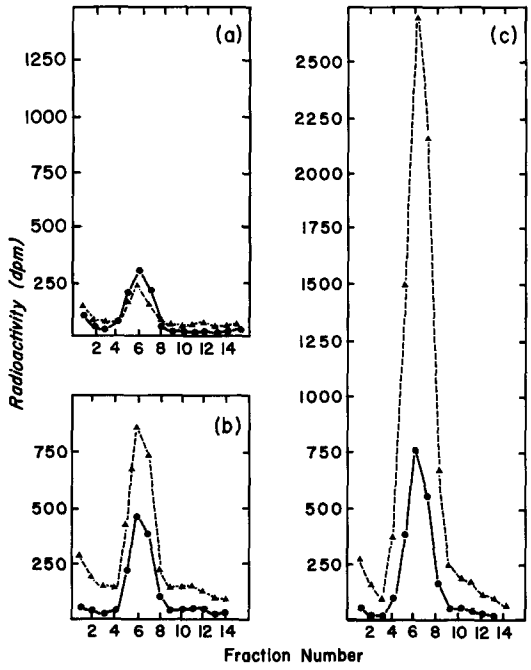


FIGURE 1 Synthesis of radioactive surface protein. 5–25% glycerol density gradients (22 h, 35,000 rpm, SW 40 rotor) of purified surface proteins. (*a*) [$^{3}$H]-leucine-labeled bacteria added 18 h after [$^{14}$C]leucine-labeled bacteria. After 4 h, paramecia were washed and protein was extracted. (*b*) 10 h chase. (*c*) 24 h chase. The surface protein is found only in the major peak: ●——●, $^{14}$C; ▲——▲, $^{3}$H.

elongation at the ribosomal level, was added to aliquots of cells at the same time that [$^{3}$H]leucine bacteria were added as chase. The synthesis of $^{14}$C- as well as $^{3}$H-labeled protein should be reduced if both the initial and chase labels are being utilized via the ribosomes.

The first noteworthy observation was that the incorporation of chase label into surface protein was inhibited more strongly by puromycin than was incorporation into TCA-precipitable protein (Table IV). Also, most significantly, even though chase label was strongly affected, the initial label; whether from cultures exhibiting increased amounts of surface protein or from those with constant amounts, is often unaffected by puromycin (Table V; also see Fig. 2). This suggests that under conditions in which there is turnover (i.e., no net protein synthesis) the original [$^{14}$C]leucine may be recycled in some form through a nonribosomal system. This is supported also by findings from cultures with an increased amount

TABLE III

*Change in Specific Activities of Newly Synthesized ($^{3}$H) Surface and Nonsurface Proteins*

| Experiment | Time after chase | Surface proteins | | Nonsurface proteins | |
|---|---|---|---|---|---|
| | | Sp act | % change | Sp act | % change |
| | *h* | | | | |
| 2671-12 | 5 | 36 | | 242 | |
| | 10 | 76 | 105 | 468 | 93 |
| 2471-97 | 5 | 19 | | 109 | |
| | 10 | 48 | 153 | 164 | 50 |
| 2971-12A | 5 | 18 | | 113 | |
| | 10 | 53 | 194 | 134 | 19 |
| 3471-12 | 4 | 26 | | 145 | |
| | 9 | 67 | 158 | 327 | 126 |
| 3471-14 | 4 | 9 | | 138 | |
| | 9 | 54 | 500 | 225 | 63 |

Specific activity is in terms of disintegration per minute per microgram protein. Nonsurface protein refers to protein precipitated by TCA from cell bodies and debris remaining after surface protein has been extracted.

of surface protein. Here, too, there was no dilution in the concentration of $^{14}$C-containing protein even though synthesis was going on and the $^{14}$C incorporation was insensitive to puromycin.

## DISCUSSION

From these studies of surface protein synthesis; indirect as they were due to the use of bacteria to introduce radioactive amino acids, two conclusions can be reached: (*a*) The protein has a high turnover rate compared with the average of other cell proteins. The evidence to support this is the rate of increase in the specific activity of the surface protein (over 4 or 5 h) compared with the change in specific activity of nonsurface (TCA precipitable) protein, and the greater inhibition of surface protein synthesis by puromycin. (*b*) Some new protein is made either from partially intact protein or from pools of precursors. This synthesis or aggregation of polypeptides does not proceed via the ribosomal pathway. Several experiments support this conclusion. For example, the original $^{14}$C-labeled protein maintains or increases its specific activity level during both turnover and net protein synthesis. Also, puromycin often has no effect on the synthesis of this "old" protein, while chase-labeled surface pro-

TABLE IV

*Effect of Puromycin on Synthesis of Surface and Nonsurface Protein*

| Experiment | Time after chase | Puromycin | Sp act of surface protein | % change | Sp act of nonsurface protein | % change |
|---|---|---|---|---|---|---|
| | *h* | | | | | |
| 2671–14 | 5 | – | 16 | | 87 | |
| | 5 | + | 6 | −63 | 71 | −15 |
| | 10 | – | 67 | | 240 | |
| | 10 | + | 34 | −49 | 109 | −55 |
| 2971–97 | 5 | – | 19 | | 109 | |
| | 5 | + | 8 | −58 | 81 | −26 |
| | 10 | – | 48 | | 164 | |
| | 10 | + | 22 | −54 | 91 | −45 |
| 3471–12 | 4 | – | 26 | | 145 | |
| | 4 | + | 9 | −65 | 122 | −16 |
| | 9 | – | 67 | | 327 | |
| | 9 | + | 29 | −57 | 208 | −36 |
| 3471–14 | 4 | – | 9 | | 138 | |
| | 4 | + | 9 | 0 | 134 | −3 |
| | 9 | – | 54 | | 225 | |
| | 9 | + | 29 | −46 | 196 | −13 |

Puromycin was obtained in the form of puromycin hydrochloride from ICN Nutritional Biochemicals Div., International Chemical and Nuclear Corp., Cleveland, Ohio. The concentration used was 4 mg/$10^6$ paramecia. Specific activity is in terms of disintegration per minute per microgram protein of the chase ($^3$H) label.

tein and TCA-precipitable protein are strongly inhibited.

The existence of such an accessory mode of protein "synthesis" would largely explain the low specific activity of surface proteins compared with TCA-precipitable protein when other data indicate a higher rate of turnover for the surface protein. New protein would be the result of synthesis with [$^3$H]leucine (through a ribosomal pathway) together with assembly of previously made subunits into complete antigen. Preliminary experiments extending the time of incubation to 48 h (and utilizing a second "chase" of cold bacteria) support the view that the specific activity of the surface protein ultimately will at least equal that of the nonsurface protein. The data also support the view that $^3$H label replaces $^{14}$C-labeled material.

The calculations comparing the observed with the expected specific activities of the original label if dilution were effective (Table II) were based on the amount of antigen in the peak of the gradient after two lengths of time. Unfortunately, due to the problems of handling small volumes of material; especially during dialysis,

TABLE V

*Effect of Puromycin on Conserved and New Surface Protein Synthesis*

| Experiment | Chase ($^3$H) | Puromycin | Protein in peak | $^{14}$C | Sp act ($^3$H) |
|---|---|---|---|---|---|
| | *h* | | *μg* | | |
| 2671–14 | 5 | – | 85 | 55 | 16 |
| | 5 | + | 74 | 57 | 6 |
| | 10 | – | 113 | 51 | 67 |
| | 10 | + | 70 | 47 | 34 |
| 2471–97 | 5 | – | 44 | 17 | 19 |
| | 5 | + | 33 | 19 | 8 |
| | 10 | – | 49 | 18 | 48 |
| | 10 | + | 50 | 18 | 22 |
| 2471–12 | 5 | – | 66 | 26 | 33 |
| | 5 | + | 81 | 24 | 11 |
| | 10 | – | 97 | 22 | 44 |
| | 10 | + | 54 | 26 | 41 |
| 2671–12 | 5 | – | 128 | 34 | 36 |
| | 5 | + | 147 | 38 | 17 |
| | 10 | – | 129 | 30 | 76 |
| | 10 | + | 162 | 43 | 34 |
| 3171–14–1 | 4 | – | 15 | 36 | 14 |
| | 4 | + | 19 | 27 | 7 |
| | 8 | – | 28 | 30 | 30 |
| | 8 | + | 26 | 37 | 20 |
| 3171–14–3 | 4 | – | 7 | 49 | 2 |
| | 4 | + | 16 | 28 | 2 |
| | 8 | – | 12 | 24 | 18 |
| | 8 | + | 12 | 33 | 10 |
| 3471–12 | 4 | – | 36 | 45 | 26 |
| | 4 | + | 23 | 37 | 9 |
| | 9 | – | 32 | 35 | 67 |
| | 9 | + | 23 | 41 | 29 |
| 3471–14 | 4 | – | 30 | 51 | 9 |
| | 4 | + | 25 | 54 | 9 |
| | 9 | – | 30 | 63 | 54 |
| | 9 | + | 16 | 38 | 29 |

For details, see legend of Table IV.

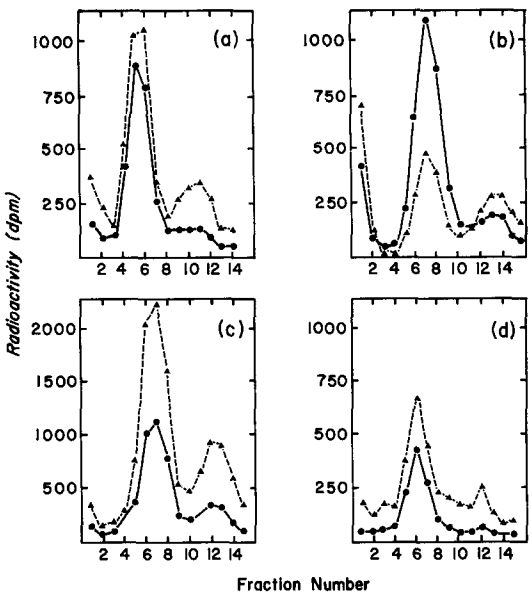


FIGURE 2 Effect of puromycin on surface protein synthesis. 5–25% glycerol density gradients (22 h, 35,000 rpm, SW 40 rotor) of purified surface protein. (*a*) [$^{3}$H]leucine-labeled bacteria added 18 h after [$^{14}$C]leucine-labeled bacteria. After 3 h, paramecia were washed and protein was extracted. (*b*) 3 h chase in presence of puromycin. (*c*) 9 h chase. (*d*) 9 h chase in presence of puromycin.

considerable variability was encountered in the amounts of antigen placed on individual gradients. However, apparently this difficulty was not serious enough to invalidate the conclusions drawn from Table II with reference to the conservation of old label since in all ten experiments conservation was indicated. Furthermore, the averages of the specific activities of chase label given in Table I are much higher for those clones in which surface protein was increasing than for those clones in which the amount of surface protein was unchanged. This also suggests that for many purposes the variables in isolation of the protein are not unmanageable.

One can speculate on the source of the $^{14}$C which became incorporated into complete antigen without passing through a puromycin sensitive system. Were there pools of previously synthesized subunits within the paramecia or were such subunits free in the medium? Should the postulated subunits exist, did they originate as a breakdown product of old surface protein and thus were being recycled, or were these "excess" subunits that during the chase were being assembled into complete antigens?

The possibility that subunits may be free in a cell's medium may have a bearing on the observation that culture fluid from one clone can induce changes in the kind of surface proteins made by another clone (9). That the phenomena described here may have a wider significance, also, may become evident from an appreciation of similarities between these surface proteins and immunoglobulins. Both types of molecules are composed of subunits held together by disulfide linkage (19, 14). Each can display a number of different specificities (many more, of course, in the case of antibodies) (1). In heterozygous cells, only one allele may be expressed (7, 10, 11). And, finally, the amino acid compositions are remarkably similar, especially the ratio of threonine:leucine compared with most proteins, and the relatively large amount of cysteine (3, 17, 20). It may perhaps be going too far to suggest an evolutionary relationship between these two kinds of macromolecules, but the current findings at least suggest that there may exist a counterpart to the light chains found free in the fluids of individuals with multiple myeloma (15). Is it possible that the pools of precursors of the *Paramecium* surface protein are in some ways analogous to Bence Jones protein? Conceivably, what has been observed here is simply one instance of a phenomenon that may occur generally among larger proteins whose subunits are linked by disulfide bonds.

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