Interrelationship between Membrane Protein Composition and Deoxyribonucleic Acid Synthesis in *Escherichia coli*†

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ABSTRACT: In an attempt to examine the role of the bacterial membrane in the process of DNA synthesis, the effect of inhibition of DNA synthesis on membrane structure has been studied. Mutants of *Escherichia coli* which are defective in initiation or replication of the chromosome had been shown to have a relative deficiency of a membrane protein complex of molecular weight *ca.* 60,000, as determined by the migration of proteins from disaggregated (1% sodium dodecyl sulfate, 40°) bacterial membrane preparations on polyacrylamide gel electrophoresis. The migration of the protein component which was deficient in mutant strains was dependent upon the temperature of disaggregation of the membrane preparations. This phenomenon was studied with greater sensitivity in the present manuscript, where the affected fraction was eluted from the gel and subjected to repeat electrophoresis; in both cases it behaved as expected from studies with the less purified membrane preparation. *E. coli* auxotrophs were starved for amino acids, in order to block initiation of new cycles of chromosomal replication. *E. coli* cells which are reinitiating DNA replication after amino acid starvation synthesize membranes which differ from the membranes of logarithmically growing cells in that they have a deficiency of a protein fraction of 60,000 molecular weight (MP60). After reinitiation has occurred in all cells of the culture, the membrane protein composition is identical with that from logarithmically growing cells which had not been starved for amino acids. Thus, during the process of initiation, membranes are synthesized which lack a protein fraction of similar molecular weight to that found deficient in mutants of *E. coli* which were affected in chromosomal synthesis. Various other techniques for inhibiting DNA synthesis were examined, in order to see whether similar membrane changes would be evoked. Thymine starvation, fluorodeoxyuridine, nalidixic acid, and hydroxyurea all led to several membrane protein alterations, the most common being at MP60. Mitomycin C was the only inhibitor of DNA synthesis which was tested which did not lead to a alteration in MP60. On the other hand, agents which inhibit cell division without affecting DNA synthesis, such as penicillin and diazouracil, did not lead to any alteration at MP60. A mutant which is unable to divide at 40°, but which synthesizes DNA normally at this restrictive temperature, had no significant membrane alterations. Thus, a correlation has been established between inhibition of DNA synthesis and a deficiency at MP60; whether the alteration in MP60 is caused by a decrease in one or several proteins, each of which may be affected differently in the several conditions examined is not known, nor is the role of the affected material in DNA synthesis understood.

In an attempt to correlate chromosomal replication with other aspects of bacterial cell division, Jacob *et al.* (1963) proposed a model which suggested that these processes could be temporally coordinated if the DNA were spatially oriented *via* a liason with the cell surface. According to this model, DNA synthesis would occur with the chromosome attached to the membrane, and subsequent membrane synthesis would separate the genetic replicas; the occurrence of septation between the DNA copies then leads to daughter cells of identical genetic constitution. Over the past several years a host of evidence has accumulated which suggests that the bacterial chromosome is attached to the cytoplasmic membrane (reviewed by Lark, 1969), but the biochemical role of the membrane in chromosomal replication has remained obscure.

We have previously found that membrane protein alterations occur in certain temperature-sensitive *Escherichia coli* mutants which are affected in DNA synthesis (Shapiro *et al.*, 1970; Siccardi *et al.*, 1971). Inouye and Guthrie (1969) have made similar observations. One class of mutants of the *dnaA* locus (near *ilv*), is unable to initiate new rounds of chromosomal replication at 40°, yet grows normally at 30° (Hirota *et al.*, 1970). A protein fraction is relatively deficient in membranes isolated from these "initiator" mutants grown under restrictive conditions, either when they are compared to isogenic wild-type strains, or with themselves grown at the permissive temperature (Shapiro *et al.*, 1970).

We studied (Siccardi *et al.*, 1971) three mutants at a second locus, *dnaB* (near *purA*), which stopped synthesizing DNA immediately upon elevation of the temperature to 40° and which appeared to be affected in the replication process itself, rather than in initiation (Hirota *et al.*, 1968). Like the *dnaA* strains, these mutants were found to have a deficiency of a membrane fraction of molecular weight *ca.* 60,000 when incubated at restrictive temperature (Siccardi *et al.*, 1971). Inouye and Guthrie (1969) have also found a membrane protein to be deficient in a second class of mutants which were temperature sensitive for chromosomal replication.

The apparent molecular weight of the protein fraction which is affected in the *dnaA* and *dnaB* mutants depends upon the conditions of disaggregation of the bacterial membrane preparations. Thus, by examining the effects of varying the temperature of disaggregation on membrane protein composition (Siccardi *et al.*, 1971), we concluded that the results were compatible with the hypotheses that the differences seen

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with disaggregation at 40° were caused by differences in the quantities of aggregate species in the membrane preparations, and that these aggregates could be further dissociated if the solubilization of the membrane preparations was at 100°. These studies were done by disaggregating intact membrane preparations at two different temperatures (Siccardi *et al.*, 1971), and many proteins migrate differently under these conditions (Figure 1). In this manuscript, we have extended these observations to more purified preparations of membrane proteins, after eluting the affected membrane species from polyacrylamide gels. With a partially purified membrane protein fraction we have obtained the same results as with the complete membranal protein mixture: a deficiency at MP60[1] in membranes of *dnaA* mutants compared to wild-type strains is changed to a difference in a fraction of about 35,000 molecular weight, when membrane protein disaggregation is at 100°. This result, and others in the recent literature (*e.g.*, Bretscher, 1971), suggest that the correlation between molecular weight and rate of migration of membrane proteins in sodium dodecyl sulfate–polyacrylamide gels may not be an absolute one, but may vary with the nature of the membranal component and the conditions of disaggregation. For this reason, we have elected to use the somewhat more vague terminology, MP60, to identify fractions of interest.

In addition, the present manuscript is concerned with the effects of certain nonmutational inhibitions of DNA synthesis on the membrane protein composition, in an effort to define more clearly the interrelationship of DNA synthesis and membrane structure. We begin by considering membrane alterations seen with cells which are reinitiating synthesis of DNA after amino acid starvation (Maaloe and Hanawalt, 1961; Lark *et al.*, 1963). These experiments are an attempt to discover whether the changes previously seen with the *dnaA* mutants (Shapiro *et al.*, 1970) were caused by the absence of a specific initiation and/or replication complex from the membranes of mutant cells grown at restrictive temperature. The other studies in this paper test the effects on the composition of *E. coli* membranes of various growth conditions in which DNA synthesis or cell division is inhibited, in an effort to see what secondary membranal changes may be evoked by primarily halting these processes. A problem with the interpretation of the latter experiments is that many of the agents used, although relatively specific, have pleiotropic effects and it is thus difficult to attach too much significance to observed membranal alterations. Inouye and Pardee (1970) have analyzed the effects on membranal composition of several agents which inhibit DNA synthesis, and have found specific alterations as a result of inhibiting DNA synthesis or cell division. In this manuscript, we find similarly that alterations in membrane composition do occur when DNA synthesis is inhibited, and that the nature of these alterations is related to the inhibition technique employed. We have found no consistent membrane protein compositional change when cell division is inhibited by several different mechanisms.

Experimental Section

Bacterial Strains. The following strains of *E. coli* K12 were employed in these studies: CR 34, *thr leu* lac_Y λ^S *thy* F^-; PA 505, met_A *his arg pro* lac_Y *xyl* mal_A *mtl ara* gal_B str^r λ^r F^-; CRT4614, *thy leu* str^r lac_z *dnaA;* and CRT4615, a temperature-resistant derivative otherwise isogenic with CRT4614 (Shapiro *et al.*, 1970); $P_{4\times8}$ *met* and a temperature-sensitive filament forming derivative, containing the PAT84 mutation, $P_{4\times8}$ *leu* ts_{84}, were all generous gifts of Y. Hirota; $P_{4\times8}$ *leu* was a wild-type revertant of the latter strain isolated in our laboratory; MX74T2 *thy* and MX74T2-ts27, containing a temperature-sensitive DNA synthesis mutation, were gifts of Dr. M. Inouye (Inouye and Pardee, 1970).

Chemicals. The drugs have been purchased or received as a gift from the following companies: nalidixic acid (gift of Sterling Wintrop through the courtesy of Dr. Goss), fluorodeoxyuridine (gift of Hoffman-LaRoche), hydroxyurea (Sigma), 5-diazouracil (Sigma); penicillin (Squibb), and phenethyl alcohol (Eastman). The radioactive compounds were from New England Nuclear, and their purity was ascertained as previously described (Shapiro *et al.*, 1970).

Media and Growth Conditions. The culture medium used in these experiments was $63B_1$ (Kohiyama *et al.*, 1966) supplemented with 40 μg/ml of thymine, 20 μg/ml each of required amino acids, and 0.4% glucose. Thymine and amino acid starvation experiments were performed by filtering the cultures onto 47-mm Millipore filters (1.2 μ pore size), rinsing with unsupplemented $63B_1$, and resuspending the bacteria in $63B_1$ containing all but the supplement of interest for the starvation experiment. Chase experiments were performed by diluting the bacteria fourfold into fresh medium containing a tenfold excess of isotopically cold material.

In a typical experiment, late log-phase cells were diluted into fresh medium at an OD_{600} = 0.05 and grown aerobically to OD_{600} = 0.2 before starting the experiments; exponential growth commenced immediately upon dilution. Radioisotopes were employed in proportions (60 μCi of ^{14}C and 300 μCi of ^{3}H per 100 ml of culture) which gave comparable number of counts per minute under our scintillation counting conditions. When cultures were to be labeled for two generations of growth, the isotopes were added at OD_{600} 0.2; when labeling was for only 30 min, they were added at OD_{600} 0.4. The generation time in the supplemented minimal medium was 75 min.

Membrane Purification and Disaggregation. After growth and labeling of the cultures, they were harvested by pouring onto crushed ice, centrifuging, and washing twice with 0.05 M Tris-HCl (pH 8.1). All subsequent procedures were at 4° unless stated otherwise. The cultures to be compared (labeled with [^{14}C]leucine and [^{3}H]leucine, respectively) were then mixed in 1:1 proportions (w/w) and membrane vesicles were purified as previously described (Shapiro *et al.*, 1970). A typical membrane preparation from 100 ml of mixed bacterial preparation (50 ml from each culture to be compared; OD_{600} 0.8) contains 1–1.6 mg of protein, as estimated by the method of Lowry (Layne, 1957) using bovine serum albumin as standard, and 0.5 × 10^7 to 5.0 × 10^7 cpm per mg of protein of either isotope, with our counting conditions.

Disaggregation was either at 40° in 1% sodium dodecyl sulfate containing 5 mM β-mercaptoethanol, or at 100° in the same solution, sealed under nitrogen to prevent oxidation and evaporation of the samples. As previously described (Siccardi *et al.*, 1971), either condition leads to over 99% solubilization of membrane radioactivity.

All critical experiments were repeated with the isotopic tracers reversed, in order to control for possible artifacts of the double-labeling techniques employed. In addition, control experiments, in which the same culture was divided into two

[1] MP60 is used to refer to a membrane protein fraction which has a molecular weight approximately 60,000. In this operational definition, nothing is implied about the number of molecular components which comprise MP60, nor even that it represents a single complex species. The terminology is used merely to facilitate reference to areas of the electrophoretograms being discussed.

portions, grown on either [^{14}C]leucine or [^{3}H]leucine under identical conditions, then subjected to purification and analysis by our techniques, showed that the apparent membrane protein composition was independent of the isotopic tracer employed.

Electrophoresis and Fractionation of the Gels. Conditions of electrophoresis were as previously described (Shapiro *et al.*, 1970) in 0.5 × 12.0 cm gel columns. Runs were performed with a Canalco Model 12 apparatus at 10–12 mA/tube and stopped when the tracking dye (Bromophenol Blue) had traveled 11 cm. The gels were fractionated with the aid of an Autogeldivider (Savant), the gel fragments being eluted with 0.1% sodium dodecyl sulfate directly into scintillation vials; 10 ml of Instagel (Packard) emulsifier–scintillant was added to each vial, and the vials were tightly capped and shaken at 37° for 3–5 hr to extract all of the radioactivity from the gel particles.

Scintillation Counting and Data Processing. A Beckman Model LS 250 was used with window settings adjusted to keep the ^{3}H overlapping in the ^{14}C channel less than 1% and the ^{14}C spillover into the ^{3}H channel around 9.5%. With these counting conditions, ^{3}H and ^{14}C efficiencies were 7 and 35%, respectively. The data were analyzed with a Fortran modification of the program we previously used (Siccardi *et al.*, 1971); most of the figures in this paper were generated by a Calcomp plotter.

Results

Effect of Temperature on the Disaggregation of Membrane Protein Preparations. As we have previously discussed, the membrane protein composition seen on sodium dodecyl sulfate–polyacrylamide gel electrophoresis is dependent upon the temperature of disaggregation of the purified membrane vesicles (Siccardi *et al.*, 1971). This was previously demonstrated by double-labeling techniques, but is equally apparent in stained gels (Figure 1). It is clear from this figure that, upon disaggregation at 100°, there is an apparent decrease in MP60 (and to a lesser extent, MP30), and an apparent increase in protein identified as MP38, when gels are compared with otherwise identical ones for which membrane disaggregation was at 40°. In our previous work, we found that the protein component which was deficient in *dnaA* mutants (defective in the initiation of DNA synthesis) underwent a similar shift in mobility when disaggregation was at 100°. Since not all of the protein at the MP60 area changed in mobility if disaggregation was at 100°, we felt that this change in migration of the deficient component might prove to be an aid in its purification. To pursue this, we isolated the protein around the MP60 area from gels of membranal material disaggregated at 40°, and reran the gels, after disaggregating a portion of the isolate at 100°.

The results of such an experiment are shown in Figure 2. Figure 2A shows the membrane protein composition of mutant and wild-type membranes from strains isogenic aside from a *dnaA* mutation, and the results are as we reported previously (Shapiro *et al.*, 1970). The principal alterations are a relative deficiency in the mutant membranes of an MP60 protein component and a relative increase of an MP30 component, when mutant and wild-type strains are grown at the restrictive temperature, 41°. When the MP60 material is eluted from the gels (fractions 35–50) and rerun, the same difference is seen as in the complete membrane preparation (Figure 2B), but now the excess of MP60 protein in the wild-type accounts for a proportionately larger percentage of the protein put on the gel. When the eluate from Figure 2A is disaggregated at 100° before repeat electrophoresis, however, there is a conversion to smaller molecular weight material (Figure 2C), with the material in excess in the wild-type moving as if it had a lower molecular weight, in complete accord with our previous observations (Siccardi *et al.*, 1971). The material in excess in the wild-type, *i.e.*, the MP60 component in Figure 2A, accounts for some 3% of the membrane protein on the gel; in Figure 2B,C, it accounts for some 9% of the total protein on the respective gels, in agreement with the observation that the protein in fractions 35–50 of Figure 2A accounted for about one-third of the total protein. Thus, by eluting and reexamining an altered membranal component, we have increased the relative percentage of this component in the protein mixture, while at the same time showing that the behavior of the component is as predicted from our earlier studies with the cruder system. This experiment lends support to the use of the somewhat cruder system in screening for membranal alterations induced by perturbations of growth conditions.

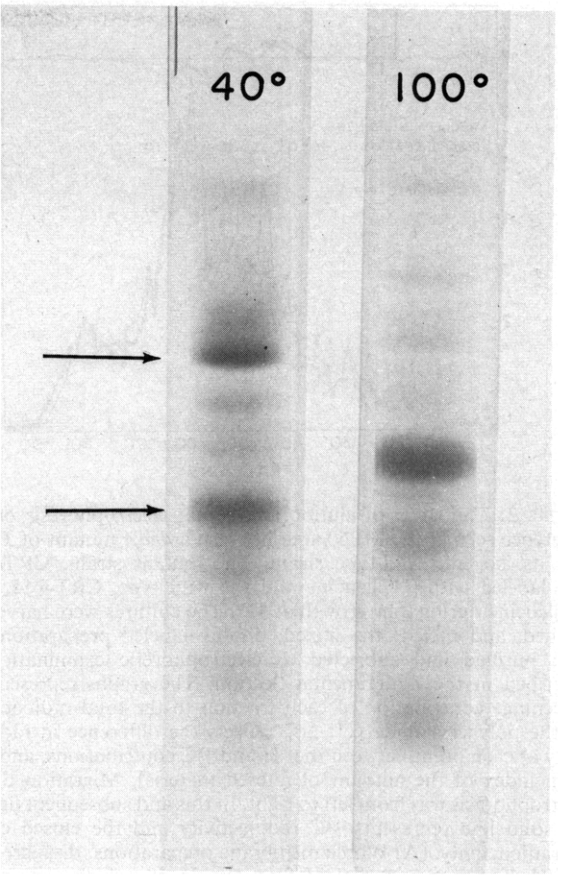

FIGURE 1: A comparison between protein patterns in polyacrylamide electrophoresis seen when membranes are disaggregated at 40 or 100°. Membranes from CRT 4615 were disaggregated in 1% dodecyl sulfate at 40 or 100°, then were subjected to electrophoresis and stained with Coomassie Blue dye. The upper arrow indicates the MP60 area; the lower arrow the MP 30 area.

Amino Acid Starvation and Initiation of DNA Synthesis. Starvation of amino acid auxotrophs of *E. coli* is a nonlethal treatment which stops protein synthesis immediately and allows a limited amount of DNA synthesis to continue. Maaloe and Hanawalt (1961) hypothesized that the residual DNA synthesis was caused by the completion of already initiated replication, but that initiation of new rounds of

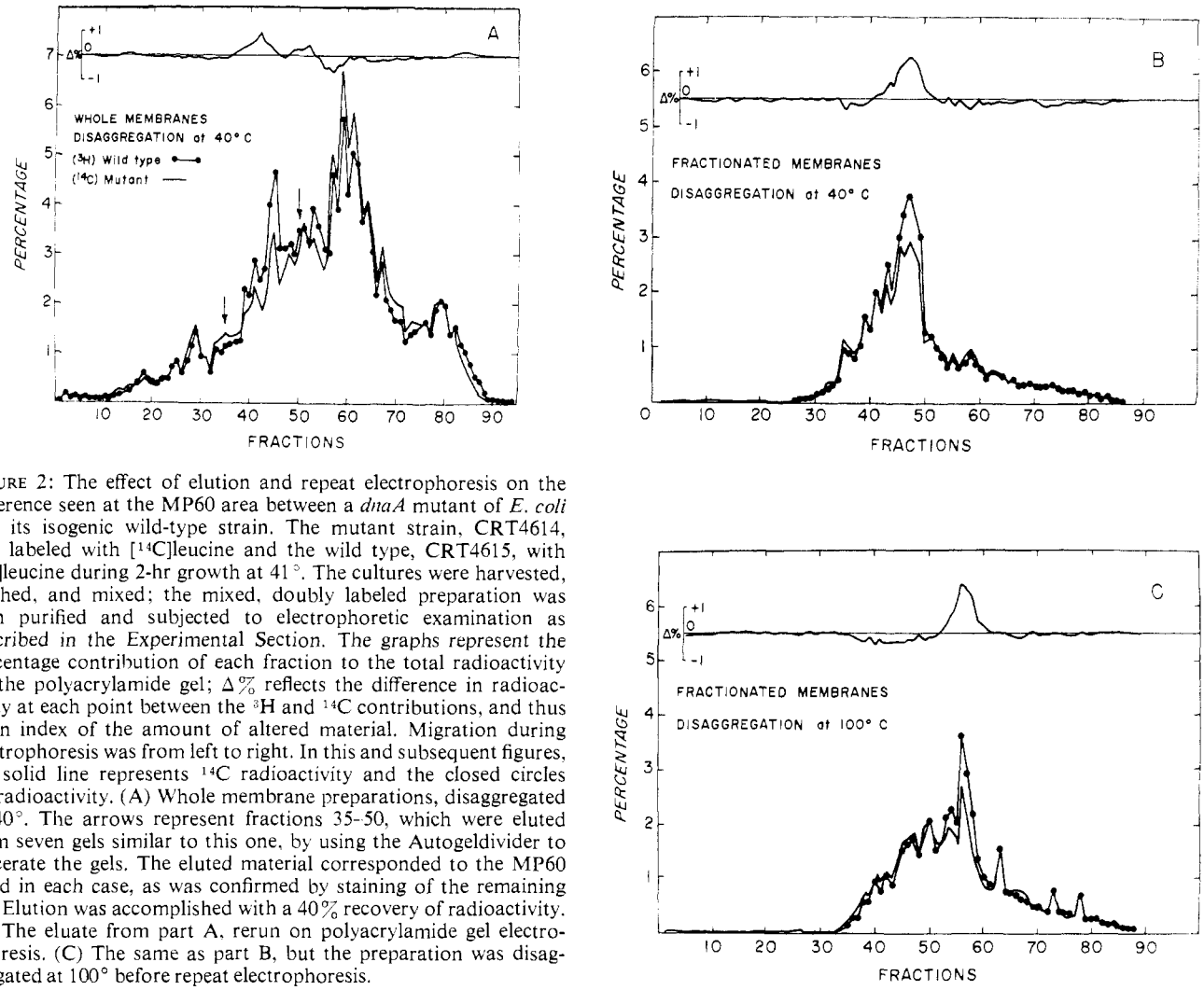

FIGURE 2: The effect of elution and repeat electrophoresis on the difference seen at the MP60 area between a *dnaA* mutant of *E. coli* and its isogenic wild-type strain. The mutant strain, CRT4614, was labeled with [^{14}C]leucine and the wild type, CRT4615, with [^{3}H]leucine during 2-hr growth at 41°. The cultures were harvested, washed, and mixed; the mixed, doubly labeled preparation was then purified and subjected to electrophoretic examination as described in the Experimental Section. The graphs represent the percentage contribution of each fraction to the total radioactivity on the polyacrylamide gel; $\Delta\%$ reflects the difference in radioactivity at each point between the ^{3}H and ^{14}C contributions, and thus is an index of the amount of altered material. Migration during electrophoresis was from left to right. In this and subsequent figures, the solid line represents ^{14}C radioactivity and the closed circles ^{3}H radioactivity. (A) Whole membrane preparations, disaggregated at 40°. The arrows represent fractions 35-50, which were eluted from seven gels similar to this one, by using the Autogeldivider to macerate the gels. The eluted material corresponded to the MP60 band in each case, as was confirmed by staining of the remaining gel. Elution was accomplished with a 40% recovery of radioactivity. (B) The eluate from part A, rerun on polyacrylamide gel electrophoresis. (C) The same as part B, but the preparation was disaggregated at 100° before repeat electrophoresis.

chromosomal replication required protein synthesis. Lark *et al.* (1963) similarly found that amino acid starvation leads to the arrest of chromosomal replication at a discrete, heritable chromosomal locus; since then several lines of evidence have supported the hypothesis that protein synthesis is required for the initiation of chromosomal replication (reviewed by Lark, 1969). Because our previous results had shown that *E. coli* mutants affected in initiation of DNA synthesis had an altered membrane protein composition (Shapiro *et al.*, 1970; Figure 2), we were interested in whether similar membranal alterations accompanied other defects in initiation of chromosomal synthesis. For this reason, we examined the effects on the membrane protein composition of *E. coli* cells of starvation for, and readdition of, amino acids. These metabolic perturbations do not have the pleiotropic sequelae of the DNA synthesis inhibitors employed below.

Figure 3 shows the effect of amino acid starvation and subsequent restoration on the membrane protein composition of an auxotrophic strain. When cells are labeled for 3 hr, during the exponential growth phase, and one culture is transferred to medium lacking required amino acids, there is no difference between the membrane protein composition of the starved culture and the culture harvested immediately after labeling (Figure 3A). However, after 150 min of amino acid deprivation, when DNA synthesis has completely stopped in this strain, the subsequent readdition of amino acids is accompanied by several changes in membrane protein composition (Figure 3B). Membranes from cells labeled during the first hour after amino acid supplementation to the culture show a deficiency of an MP60 protein fraction and increases of several other protein fractions. This membrane protein alteration is not strain specific, since, when CR 34, another strain of *E. coli* K12 of completely different genetic background, is deprived of amino acids for enough time to allow DNA synthesis to come to a complete halt (150 min) there is a similar major alteration in the membrane protein composition during the reinitiating phase, with a decrease in MP60 in a reinitiating culture compared to a logarithmically growing culture (Figure 4A).[2] There are other small membrane protein changes seen with both strains, but the one at the 60,000 molecular weight area is the one which is absolutely reproducible in different strains and in all repeat experiments of this type.

Upon readdition of amino acids to deprived cultures of

[2] An occasional difficulty with the gel fractionation apparatus we employ is shown in this figure. Fractions may contain variable quantities of gel (*e.g.*, fractions 32-45 in Figure 4A). All of the protein is eluted from the gel before counting and we have never noticed differential quenching of our fractions; thus the double-labeling technique we employ serves as a control for this problem. We are now using an improved head for the Savant Autogeldivider, with less dead space between the screen which fractionates the gel and the buffer which elutes the fractions, and this has obviated the problem seen with earlier experiments.

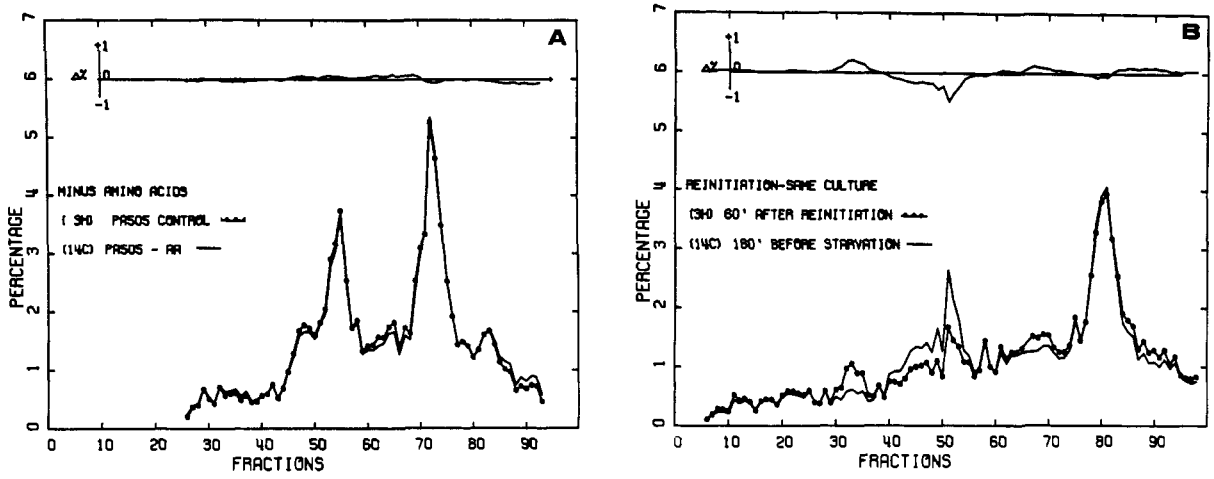

FIGURE 3: The effect of amino acid starvation and subsequent resupplementation on the membrane protein composition of *E. coli*. Cultures of PA505 were examined while growing exponentially at 37°; in part A a culture was labeled for 3 hr with [^{14}C]leucine, then transferred to medium lacking the required amino acids for 150 min; the control culture was labeled with [^{3}H]leucine for 3-hr exponential growth with no subsequent starvation period. The cultures were harvested, washed, and mixed, and the mixed, doubly labeled preparation was purified and subjected to examination as described in the Experimental Section. In part B the culture was labeled with [^{14}C]leucine as in part A, then subjected to amino acid deprivation in the same fashion; however, after the 150-min starvation period, the required amino acids were resupplemented to the medium, along with [^{3}H]leucine, and labeling proceeded for an additional 60 min. The fractions in part B were of smaller volume, to increase the resolution between peaks.

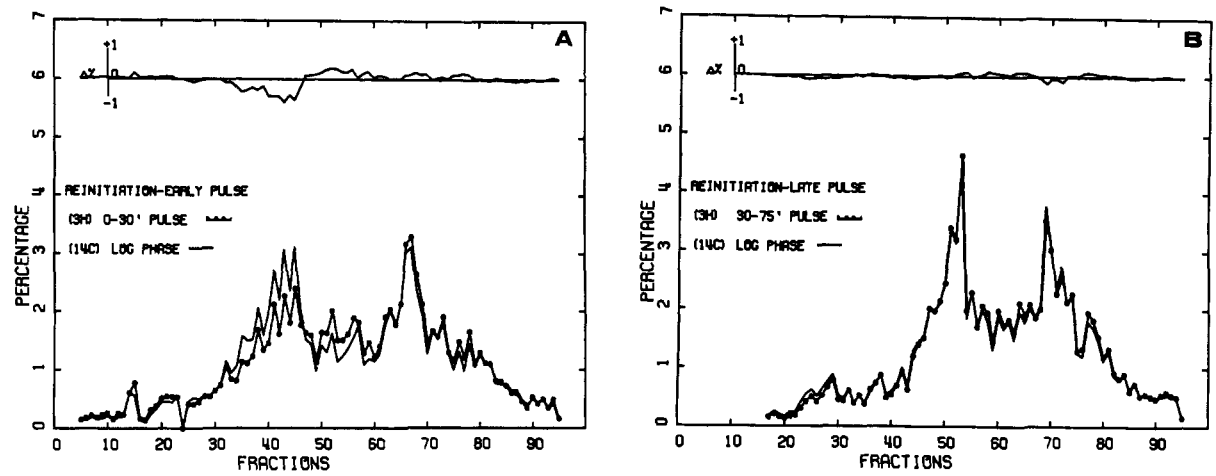

FIGURE 4: The effect of labeling reinitiating cultures at varying times after readdition of amino acids. Strain CR 34 growing exponentially at 37° was starved for amino acids for 150 min. Upon subsequent readdition of the required amino acids, part of the culture was labeled with [^{3}H]leucine from 0 to 30 min after starvation (A); the other part of the culture was labeled with [^{3}H]leucine from 30 to 60 min after starvation (B). The control culture was an exponentially growing culture of CR 34, labeled with [^{14}C]leucine for 30 min.

either strain, there is a 30-min lag period before DNA synthesis resumes the rate characteristic of logarithmically growing control cultures; cell growth, on the other hand, resumes immediately at the logarithmic value. The lag in DNA synthesis probably reflects nonsynchronous reinitiation by the culture. To investigate whether the membrane protein difference was generated only during the period before DNA synthesis resumed in all cells, the experiments in Figure 4 were performed. Figure 4A shows the membrane protein composition of cells labeled only during the period before DNA synthesis achieves its maximal rate.[2] There is, as mentioned before, a relative absence of MP60 in the membranes of reinitiating cultures, as compared to logarithmically growing cells. If labeling is confined to the period after DNA synthesis reaches its maximal rate, however, the composition of the membrane proteins in the resupplemented culture is the same as in the logarithmically growing culture (Figure 4B). These data indicate that, whatever is responsible for the membranes being synthesized with an abnormal composition after amino acid starvation, the abnormal membrane synthesis is restricted to the time before DNA synthesis reaches its maximal rate (*i.e.*, before initiation is completed in all cells in the culture). To examine the possibility that the protein components which constitute the deficient 60,000 molecular weight material were synthesized during the first 30 min after readdition of amino acids, but not integrated into the membrane structure during this time, we performed an isotopic chase experiment. When cells labeled as in Figure 4A are subjected to a "chase" with isotopically cold leucine for 150 min, they preserve, almost quantitatively, the membrane protein difference shown in Figure 4A. This experiment is compatible with there being no

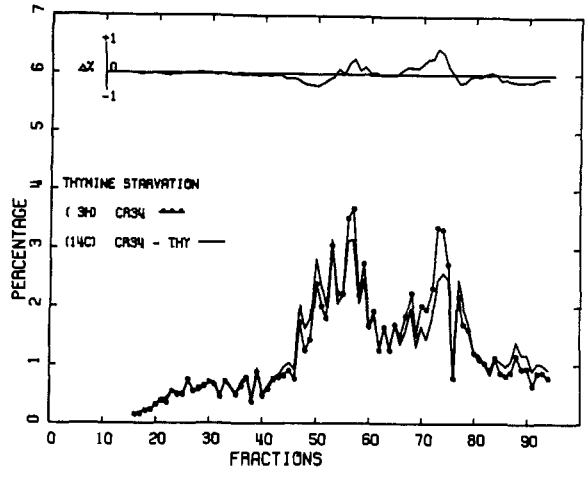

FIGURE 5: The effect of thymine starvation on membrane protein composition. Cultures of CR 34 growing exponentially at 30° were harvested by filtration and resuspended in supplemented minimal medium containing no thymine, and then labeled with [^{14}C]leucine for 150 min. The control was CR 34 growing exponentially on the same medium containing thymine, and was labeled with [^{3}H]leucine for the same period of time.

relative excess of cytoplasmic precursor during the first 30 min, which then could have integrated into the membrane at a later period. A similar chase experiment, in which cells labeled during the latter period of resupplemented growth (Figure 4B) were "chased" with 150-min growth on cold leucine, showed the same pattern as in Figure 4B, as expected. Thus, the data indicate that the membrane protein material synthesized after readdition of amino acids to a starved culture is composed of all of the components present in logarithmically growing cells with the exception of a component of molecular weight 60,000. However, after reinitiation of chromosome synthesis has occurred in all cells of the culture, the membrane proteins synthesized are identical with those of logarithmically growing cells.

Effects of DNA Synthesis Inhibition on Membrane Protein Composition. Because we had found membrane changes accompanying DNA synthesis inhibition by several different mechanisms, we were interested in discovering whether membrane alterations were concomitant with any condition which resulted in the cessation of DNA synthesis or cell division. To do this, we decided to explore the effects on membranal structure of starvation of auxotrophs for thymine, or of addition of chemical agents which are known to interfere with DNA synthesis. While we were preparing these results for publication, Inouye and Pardee (1970) reported membranal alterations accompanying a variety of conditions in which DNA synthesis is inhibited. The difficulty with interpreting the results of such an experimental approach is that even the most specific methods used to inhibit DNA synthesis often cause secondary effects on DNA degradation, nucleotide pool size, and even on RNA and protein synthesis. However, the experiments reported below had been undertaken in an attempt to see whether changes in MP60 such as seen with the DNA synthesis mutants and with cultures reinitiating after amino acid deprivation, were also associated with other disturbances of DNA or cellular metabolism.

One of the techniques we used (as did Inouye and Pardee, 1970) to inhibit DNA synthesis was the starvation of auxotrophic cells for thymine. While this results in inhibition of DNA synthesis, it also results in unbalanced growth and thymineless death (reviewed by O'Donovan and Neuhard, 1970), as well as in alterations in nucleotide pools and excretion of nucleotide precursors into the medium. Thus, this direct method of inhibiting DNA synthesis has several sequelae which reflect profound disturbances in cellular metabolism. As shown in Figure 5, starvation for thymine leads to the synthesis of membranes of abnormal composition. Although the differences between membranes from starved and exponentially growing cells are rather small, there are three alterations which were reproducible in several repeat experiments in which the isotopes used during growth were reversed. Thymine-deprived cells have a deficit of protein on the light side of the MP60 peak, but an excess of protein slightly heavier than MP60; in addition, there is a deficiency of material of molecular weight approximately 30,000 which varies in magnitude from one experiment to another.

Thus, even with this rather direct mechanism for the inhibition of DNA synthesis we see multiple membrane changes, similar but not identical with ones seen in the previous cases, where mutants affected in DNA synthesis were examined. When we examined a variety of other agents, we likewise obtained results compatible with their being membrane alterations accompanying inhibition of DNA synthesis, the most common being a deficiency of protein in the MP60 peak; nonetheless, there were often other alterations seen. Rather than showing all of the data, the results of these experiments with respect to the MP60 and MP30 areas of the electrophoretogram, which were the areas of interest in the mutants previously studied and in the amino acid starvation experiments (Figures 3 and 4), are summarized in Table I. Specific information about these experiments is included in the text below.

We studied the effects of fluorodeoxyuridine, mitomycin C, nalidixic acid, and hydroxyurea on membrane protein composition. FUDR[3] induces thymineless death in bacteria, probably by inactivating thymidylate synthetase (Cohen *et al.*, 1958). However, since FUDR also interferes with RNA metabolism, it has metabolic effects beyond those seen with thymine deficiency. Fluorodeoxyuridine, at a concentration of 150 μg/ml, inhibited DNA synthesis immediately, led to degradation of intracellular DNA, and also inhibited growth by 70%, indicating pleiotropic effects in the strain of *E. coli* (CR 34) which we employed. The membrane alterations attendant upon administration of FUDR were a deficiency at MP60 and an increase of material of molecular weight approximately 30,000. In addition, the FUDR-treated cells had a relative deficiency of material migrating as MP80 or greater, and an increase in very low molecular weight protein. Mitomycin C is an agent which inhibits DNA synthesis (Shiba *et al.*, 1959), probably by causing covalent cross-links in complementary DNA strands (Iyer and Szybalski, 1963). At a concentration of 15 μg/ml, mitomycin C inhibited DNA synthesis completely in CR 34, and inhibited growth about 50%. Under these conditions there was only a slight effect at MP60, but a rather marked inhibition of the synthesis of a 30,000 molecular weight membrane protein component. These results are reproducible, though small in magnitude; very little (or no) decrease in MP60 and a significant decrease in MP30 accompany inhibition of DNA synthesis by mitomycin C.

Nalidixic acid is an agent which inhibits DNA replication in *E. coli* by an unknown mechanism, but which is reported

[3] Abbreviations used are: FUDR, fluorodeoxyuridine; DZU, diazouracil.

TABLE I: Summary of the Changes Seen in Membrane Proteins as a Function of Conditions which Inhibit DNA Synthesis and Cell Division.[a]

	MP60	MP30
DnaA mutants	↓ (2/2)[b]	↑ (2/2)[b]
DnaB mutants	↓ (3/3)[b]	↑ (2/3)[b]
Reinitiation after amino acid deprivation	↓	NC
Thymine starvation	↓ ↑[c]	↓
Fluorodeoxyuridine	↓	↑ ↑[c]
Mitomycin C	NC	↓
Nalidixic acid	↓	↓
Hydroxyurea	↓	↑
Penicillin	NC	NC
5-Diazouracil	NC	NC

[a] ↑ indicates an increase of this component; ↓ indicates a decrease in this component. NC indicates no change. [b] The fractions indicate the number of different mutants at this locus which showed the difference seen over the total number of mutants examined. [c] Differences of this nature occurred on either side of the peak in question, rather than on the peak itself.

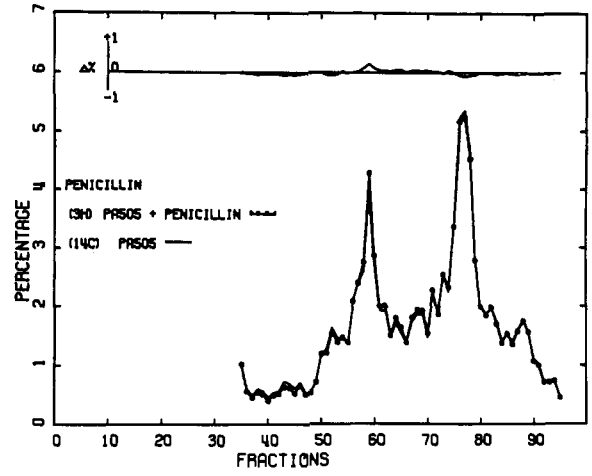

FIGURE 6: The effects of penicillin on the membrane protein composition of *E. coli* strain PA505, growing exponentially in supplemented minimal medium containing 20% sucrose (w/v), was labeled with [^{14}C]leucine for 180 min (control); another culture growing in the same medium containing 150 μg/ml of penicillin G was labeled for the same time with [^{3}H]leucine, during which time all of the cells were converted to spheroplasts.

to have little effect on RNA and protein synthesis (Goss *et al.*, 1965). At a concentration of 50 μg/ml, nalidixic acid inhibited DNA synthesis in the strain employed in these studies (CR 34) 70%, while inhibiting growth less than 25%. Under these conditions, there is a deficiency at MP60, a deficiency at MP30, and an excess of lower molecular weight material in the membranes of treated cultures when compared to control, logarithmically growing cells. The differences seen with nalidixic acid treatment were more variable from experiment to experiment than those seen with the other DNA synthesis inhibitors, in that the magnitude of the changes varied somewhat between different experiments. Hydroxyurea is bacteriostatic for *E. coli*, inhibiting DNA synthesis with little effect on RNA and protein metabolism (Rosenkranz *et al.*, 1966). It acts only at relatively high concentrations (0.1 M) by an unknown mechanism, but it is supposed to differ from other agents in not being bacteriocidal, so it may have some advantages in looking at membrane changes induced by inhibition of DNA synthesis selectively. With the strain we employed, 0.1 M hydroxyurea caused inhibition of growth to about 30% of the logarithmically growing control culture (in contrast to the 20% inhibition reported previously (Rosenkranz *et al.*, 1966) for C600 from which our strain (CR 34) was originally derived). In addition, we found complete inhibition of DNA synthesis and evidence of DNA degradation under these conditions, so, as with many of the other agents used to inhibit DNA synthesis, the specificity of the effect remains in doubt. Nonetheless, we saw a marked deficiency at MP60, and a large excess at MP30, in treated cultures. In addition, there were other nonspecific membranal alterations seen with this agent, the most significant one being a deficiency of material migrating somewhat more slowly than MP60.

Effects of Agents Which Inhibit Cell Division on Membrane Protein Composition. Since many chemical agents and mutational events which inhibit DNA synthesis also lead to membrane changes in *E. coli*, we were interested in whether *any* agent which inhibited cellular division would produce similar alterations. For this reason, we examined the effects of two chemicals, penicillin and diazouracil, which inhibit cell division without affecting DNA synthesis, in an attempt to isolate the effects of inhibition of either of these processes. In addition, we examined the membrane proteins from a cell division mutant of *E. coli* which forms filaments when growing at 40°, but continues to synthesize DNA normally.

Penicillin induces protoplast formation in *E. coli* (Lederberg, 1956); the cells lose their ability to divide, but continue synthesizing nucleic acid and protein and remain viable for a short time, as long as they are protected from osmotic lysis by 20% sucrose. We felt that such cells, with disrupted cell wall structure, would provide an interesting control on the effects of inhibiting cell division by another mechanism than was previously used. In fact, as is shown in Figure 6, cells quantitatively converted to spheroplasts by penicillin synthesize membrane of the same composition as do exponentially growing cells. It should be mentioned again that our membranes have been prepared after exhaustive treatment with lysozyme, so that the mucopeptide has been essentially removed.

When added to dividing cultures of *E. coli*, DZU blocks cellular division without grossly altering DNA synthesis or cell mass increase (Previc and Richardson, 1969). At a concentration of 2 μg/ml, DZU completely blocked cell division, while the increase in optical density was only inhibited 10%; there was no inhibition of DNA synthesis with the strain we used. Under these conditions, we saw no significant alteration at either MP60 or MP30 (data not shown), although there was a slight decrease in higher molecular weight material in the treated cultures.

In addition, we examined one of the temperature sensitive filament forming mutants of *E. coli* previously studied by Hirota and Jacob (Hirota *et al.*, 1968; Ricard and Hirota, 1969). This strain, PAT84, has been rather well characterized by these authors as being deficient in septum formation at 40°, while continuing other cellular functions and maintaining its ability to survive even after several hours at the nonpermissive

temperature. When shifted back to 30°, these cells complete septum formation and divide, even if chloramphenicol is added to the growth medium after the shift to permissive temperature (Hirota *et al.*, 1968). The latter observation made it unlikely that there would be a membrane protein deficiency at the elevated temperature, but we were interested in whether there was any membrane protein alteration in these strains. While we compared mutant and wild-type cells at the restrictive temperature, or mutant and revertant cells at the restrictive temperature, or mutant cells at the restrictive temperature with mutant cells at the permissive temperature, we obtained no significant membrane alterations, the patterns being similar to that seen with penicillin treatment (Figure 6). The strains used in these experiments are described in the Experimental Section. Thus, with three different mechanisms of inhibiting cellular division while permitting DNA synthesis to continue, we have found no significant, reproducible membrane protein alterations.

Discussion

Problems in Interpretation of Membrane Alterations. As summarized in Table I, we have found a rather good correlation between an arrest of DNA synthesis and the relative deficiency of membranal material which migrates with the MP60 band. Inouye and his collaborators (Inouye and Guthrie, 1969; Inouye and Pardee, 1970) have also made correlations between membrane composition and DNA synthesis. Before speculating on the potential significance of these results, it is important to emphasize the limitations of the experimental techniques employed. For one thing, the electrophoresis peaks probably represent contributions of many protein components which migrate together under the conditions employed, perhaps as a function of their molecular weight, although the latter point has not been established for membrane complexes. In fact, we have previously shown (Siccardi *et al.*, 1971) that disaggregation at higher temperature leads to an altered protein electrophoresis pattern, and have supported these observations in this manuscript (Figures 1 and 2). Moreover, since the differences we observe occur, for the most part, in peaks which represent 10–30% of the total membrane protein material, it is possible that any two changes in MP60 do not reflect changes in identical molecular species. Thus, the differences seen may be generated by coordinate changes in several different molecular species which migrate together. Nevertheless, it is of some interest that a rather good correlation exists between membrane protein alterations at MP60 and the cessation of DNA synthesis. The designation MP60 is merely an operational one, for the purpose of the present study, and will be refined as purification and analysis of the affected species continues. In fact, as mentioned before (Shapiro *et al.*, 1970), the designation "membrane" is also an operational one, and is used to indicate the material which is purified by our techniques and which has some properties of the bacterial membrane. Because of the limitations of our techniques, further correlation between membrane protein alterations and DNA synthesis will be best made by looking for biological activities associated with specific membrane components, a project which we are involved in now. Another reservation should be made concernining the molecular weight of the affected material. The material has been described in terms of the migration of polypeptide chains of globular proteins under the conditions of study employed here, principally for convenience in discussing our data. Since the material in the membranes is not completely disaggregated at 40°, and since disaggregation at a higher temperature (100°) might lead to hydrolysis of labile polypeptide bonds (even though the pattern is not altered for from a 5- to 60-min exposure to 100° (Siccardi *et al.*, 1971), it is impossible to be precise about the molecular weights of the species involved. Thus, there is further reason for regarding the designation MP60 as an operational one, to be changed as we know more about the structure and function of the involved protein species.

Effect of Disaggregation Temperature on Electrophoresis Pattern. As we have reported previously (Siccardi *et al.*, 1971), and have demonstrated more directly in this manuscript (Figures 1 and 2), the temperature of disaggregation makes a marked difference in the migration of certain of the protein bands seen by polyacrylamide gel electrophoresis. This may be caused by disruption of noncovalent interactions under the harsher conditions (100°) or, perhaps, by cleaving some labile covalent bond, for example, a protein–carbohydrate linkage. Proteolysis in sodium dodecyl sulfate is unlikely to lead to these differences, for the patterns seen in electrophoresis are reproducible after several days' storage, and the difference upon disaggregation at 100° is seen after only 5 min at that temperature and is not changed upon incubation for another hour. One would thus have to postulate the existence of a heat activated protease which has activity upon only a very limited population of bonds in order to imagine that proteolysis affects the membrane pattern. Nonetheless, a modification of the experiment in Figures 2B,C might provide us with a convenient two-dimensional purification step for the protein of interest in the *dnaA* mutants (the one deficient at MP60), since the difference between mutant and wild-type strains persists even after disaggregation at the higher temperature. This membrane fraction accounts for some 2–3% of the total membrane proteins, or some 0.2–0.3% of the cell protein of *E. coli*, since, as previously described, the membranes are 10-fold purified preparations (Shapiro *et al.*, 1970). By reisolating fractions 53–63 of Figure 2C, which account for some 35–40% of the protein on those gels, or 10–15% of the total membrane protein, we will have achieved a 50- to 100-fold purification over whole cells. Thus, this technique should be a useful one in aiding us to achieve our goal of purifying individual components which are altered in DNA synthesis mutants of *E. coli*. In addition, the magnification of the difference seen between mutant and wild type (Figure 2B,C) when the eluted material is examined supports the observations made on the complete membrane protein fraction.

Effect of Inhibiting Initiation of DNA Synthesis on Membrane Protein Composition. In the experiments reported in Figures 3 and 4 of this paper, strains which were reinitiating DNA synthesis after amino acid starvation were compared to exponentially growing cultures, and a deficiency in the MP60 area was found. These data indicate that the membrane proteins synthesized immediately upon readdition of amino acids to starved cultures are similar to the membrane proteins of exponentially growing cells, with the principal exception being that they contain decreased amounts of an MP60 component. Synthesis of membranes lacking this fraction persists only during the period before DNA synthesis has been reinitiated in the whole culture; after this has occurred, the membranes are identical with those from logarithmically growing cells (Figure 4). This transient deficiency in the synthesis of MP60 is not caused by nonsynchronous recommencement of protein synthesis after starvation, for the cells start growing at their maximal exponential rate, and synthesize protein at a maximal rate, immediately upon amino acid re-

addition. Thus, this deficiency may be caused by several primary mechanisms, which include: (1) the affected component may be inserted into the membrane only when DNA synthesis is initiated, perhaps as part of the initiation event *per se;* (2) synthesis of the affected material might require concomitant replication of DNA, and might not be related to initiation directly; (3) the deficiency at MP60 could be related to the insertion of a precursor which was present during the period of amino acid starvation, and in this case, there is only a coincidental temporal relationship between DNA synthesis and the deficiency of MP60. The finding that there is no large pool of precursor which can integrate later into the MP60 peak suggests that the third possibility is not a likely one during exponential growth, but does not answer the question for the period of amino acid starvation, where specific protein turnover might lead to a large pool of MP60 percursor. Although one can imagine several physiological experiments to further test the relationship of the MP60 alteration to events in the cell cycle, by using synchronously growing cells or *dnaA* mutants examined during the reinitiation which follows a shift to 30° from the restrictive temperature, the data will be more easily interpreted when we have a more specific assay for individual components which may comprise MP60.

Effect of Inhibition of DNA Synthesis on Membrane Protein Composition. From the results summarized in Table I and discussed above, it is clear that a number of different inhibitory conditions may lead to alterations in membrane composition, and that many of these alterations are similar to those seen with mutants affected in DNA synthesis or with cells during reinitiation of DNA synthesis. However, as emphasized above, the membrane changes are often pleiotropic, not being restricted to those we have seen with mutants. Thus, it is difficult to make a strong unifying hypothesis to explain our observations with these inhibitors. We have seen that many conditions which inhibit DNA synthesis, by a variety of mechanisms, can lead to a deficiency of material at MP60. Inouye and Pardee (1970) have described a deficiency of 34,000 molecular weight protein when DNA synthesis is inhibited in their mutants, or by several other mechanisms (thymidine starvation, nalidixic acid, or ultraviolet irradiation). Their conditions differ from ours in several respects. First, they have used arginine as the radioactive label, whereas we use leucine. We have shown (A. Lazdunski, unpublished results) that the pattern of protein which is seen depends upon the radioactive amino acid precursor, as might be expected. Secondly, with the exception of the experiment in Figure 3 of this manuscript, all of our data was obtained with leucine auxotrophs, in an attempt to avoid artifacts caused by pool dilution effects. Thirdly, their membrane preparation is a washed unfractionated particulate preparation of *E. coli*, whereas our preparation is made by lysis of lysozyme induced spheroplasts and is removed from other cellular debris by sucrose gradient centrifugation (Shapiro *et al.*, 1970). Inouye and Pardee (1970) have examined the effect of sucrose sedimentation on the protein pattern they obtained, and found that they are similar to those seen with a washed particulate preparation, except that more differences were apparent between the mutant and wild type in their data with the more purified preparation. Finally, they routinely disaggregate their preparations at 70° for electrophoresis, whereas we disaggregate routinely at 40°. For all of these reasons, the data are not strictly comparable. We have examined their DNA synthesis mutant using our procedures, in an attempt to correlate our respective data. Our results (A. Lazdunski, unpublished data) with their strain show a deficiency in a protein migrating as MP35, whether disaggregation is at 40° or at 70°, and this finding is in agreement with their results. In addition, however, we find that the mutant has an excess of MP30 material when compared to the wild-type strain, and that this difference is quantitatively as significant as the deficiency at MP35. Although a variety of differences in the techniques employed can account for the differences in our results, it seems best to defer a real consideration of their significance until we can discuss the properties of more purified protein fractions. We conclude, therefore, that inhibitors of DNA synthesis may lead to alterations in membrane proteins; the structures of the affected species and their role, if any, in DNA replication, remains obscure.

Effect of Inhibition of Cell Division on Membrane Protein Composition. We have found no significant change in membrane composition when cell division is inhibited by either penicillin or diazouracil. Nor have we found a difference when a temperature sensitive filament forming mutant is examined. The mutant which we employed was a reversible one (Hirota *et al.*, 1968) and could form septae when lowered to the permissive temperature in the presence of chloramphenicol, so that it was unlikely to have a deficiency of protein at the restrictive temperature; perhaps other filament forming mutants will show membranal alterations. Inouye and Pardee (1970) found that diazouracil led to a deficiency of a high molecular weight protein (α, which migrated as if it had a molecular weight of 80,000) and an increase in a fraction of about 39,000 molecular weight. Although we saw a slight deficiency of material in treated cells which might be equivalent to their α component (see Results), the differences were quite small. As mentioned above, we feel that a more meaningful interpretation of these results awaits purification of membrane components.

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Transfer Ribonucleic Acid Synthetase Catalyzed Deacylation of Aminoacyl Transfer Ribonucleic Acid in the Absence of Adenosine Monophosphate and Pyrophosphate†

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ABSTRACT: Isoleucyl-tRNA synthetase has been found to catalyze *specific* deacylation of Ile-tRNA^{Ile} in the absence of AMP or pyrophosphate (PP_i). The K_m for the deacylation reaction is about 10^{-7} M and the turnover number is ~0.8 min^{-1} at pH 7, 37°. The rate of the enzyme-catalyzed deacylation decreases sharply with decreasing pH and exhibits a Mg^{2+} dependence which is substantially different from that of reverse aminoacylation. Unacylated tRNA^{Ile} is a potent (competitive) inhibitor of the reaction. In addition, natural substrates such as ATP, isoleucine, and the adenylate analog isoleucinyl-AMP are also inhibitors, but none are completely (100%) inhibitory. On the other hand, neither AMP nor PP_i, separately, affect the rate of deacylation. When aminoacylation of tRNA^{Ile} is carried out with approximately stoichiometric concentrations of Ile-tRNA synthetase, a considerable amount of tRNA^{Ile} is shown to be deacylated without going through reverse aminoacylation. This implies that the deacylase activity can function during aminoacylation with the result that considerably more ATP may be hydrolyzed to AMP than Ile-tRNA^{Ile} is produced. A cursory examination of a few different tRNA synthetases suggests that others may also possess the deacylation activity. Possible mechanistic and physiological implications of these findings are discussed.

Aminoacylation of tRNA is generally believed to proceed in a two-step reaction sequence in which an amino acid is condensed with ATP to form an enzyme-bound aminoacyl adenylate which in turn reacts with the 2′- or 3′-terminal hydroxyl group of tRNA to yield aminoacyl-tRNA. The reactions can be written[1] as

$$E + AA + ATP \rightleftarrows E \cdot AA\text{-}AMP + PP_i \quad (1)$$

$$E \cdot AA \sim AMP + tRNA \rightleftarrows AA\text{-}tRNA + AMP + E \quad (2)$$

In the course of investigations of isoleucyl-tRNA synthetase, it was discovered that under certain conditions the enzyme can catalyze deacylation of Ile-tRNA^{Ile} *in the absence of AMP and* PP_i. The reaction is

$$E + AA\text{-}tRNA \rightleftarrows E + AA + tRNA \quad (3)$$

Interest in this reaction stems from the fact that Lagerkvist *et al.* (1966) and Yaniv and Gros (1969) had suggested earlier that the valyl-tRNA synthetase from baker's yeast and from *Escherichia coli* B also possesses a similar activity. Moreover, a cursory examination of a few different tRNA synthetases (see below) gave evidence that other synthetases may also deacylate their cognate aminoacyl-tRNAs without going through reverse aminoacylation. Therefore, eq 3 may be a rather general reaction and its mechanism and possible significance bears investigation.

We report here an investigation of the Ile-tRNA synthetase catalyzed deacylation of Ile-tRNA^{Ile} according to eq 3. The general features of the reaction are first considered, followed by attempts to establish the existence of any relationship between the site(s) involved in aminoacylation and that for deacylation according to eq 3. Finally, some consideration of the possible significance of the deacylation reaction is given.

Materials and Methods

Materials. Unfractionated *E. coli* B tRNA (Schwarz BioResearch) was enriched threefold in isoleucine acceptance activity by BD-cellulose salt gradient chromatography. The

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[1] Abbreviations used are: AA, amino acid; AA-AMP, aminoacyl adenylate; AA-tRNA, tRNA aminoacylated with AA; E, enzyme, an aminoacyl tRNA synthetase; EtOH, ethanol; Ile-ol-AMP, isoleucinyl-AMP; Ile-tRNA^{Ile}, tRNA^{Ile} aminoacylated with isoleucine; PCMB, *p*-chloromecuribenzoic acid; Phe-tRNA, phenylalanine presumably aminoacylated to phenylalanine-specific tRNA in a mixture of unfractionated tRNA; Tu, transfer factor; tRNA-CpCp, terminal adenosine-less tRNA; tRNA^{Ile}, tRNA specific for isoleucine; Val-ol-AMP, valinyl-AMP; Val-tRNA^{Ile}, tRNA^{Ile} aminoacylated with valine.