probable mechanism, however, would rely on increased synthesis and release of melanocyte-stimulating hormones (MSH) due to the loss of circulating adrenocortical hormones. A fall in circulating levels of corticosteroids would be followed by increased synthesis of corticotropin. A variety of types of MSH have now been found, and their interrelationships with corticotropin have been established (*2*). McGuiness (*3*) found increased concentrations of circulating MSH in the blood of adrenalectomized humans. From a comparative standpoint, in addition to the well-known effects of MSH on amphibia pigmentation, endogenous MSH results in a darkening of human skin color (*4*), and stress results in pigment changes in goldfish (*5*).

Adrenalectomy has been utilized as a common laboratory procedure in a wide variety of laboratory mammals during the past two decades. To the authors' knowledge, there has been no other report of such profound hair darkening following adrenalectomy in a mammal. This would seem to point to the deer mouse as having a relatively unique genetic background in this respect. This species, then, might prove a valuable tool for studying pigmentation-MSH relationships in mammals, particularly with reference to Addison's disease in humans (a result of chronic adrenal insufficiency and probably related to MSH secretion). Another possible facet of this finding is in the role of the adrenal in speciation of *Peromyscus*, one of the most common genera of rodents in North America. Since functional level of the adrenal cortex in rodents is related to population density (*6*) and a host of other environmental factors, differences in pigmentation patterns between populations and among individuals within populations could, in some cases, be due to stress-MSH effects.

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Cell-Free Protein Synthesis: Effects of Age and State of Ribosomal Aggregation

Abstract. *In cell-free extracts derived from* Streptococcus faecalis, *protein synthesis directed by endogenous messenger RNA increases as the culture ages. The increased activity is accompanied by an increase in the percentage of membrane-bound ribosomes and by a decrease in ribosomal monomers and subunits. These changes progress against a background of structural and compositional modifications in the membrane. Membrane modifications possibly related to endogenously directed protein synthesis in cell-free extracts include: (i) decreased specific activity of a membrane-associated polynucleotide phosphorylase capable of polysome degradation, and (ii) increased concentrations of certain phospholipids.*

Cell-free protein synthesis directed by exogenous polyribonucleotides is restricted to ribosomes free of native messenger RNA (*1*). In *Escherichia coli* such a situation obtains in extracts of cells harvested during the early logarithmic (log) phase of growth (*2*). Protein synthesis directed by native messenger RNA is contingent on a high complement of functional polysomes. Maximum activity for endogenously directed protein synthesis occurs in extracts of *Streptococcus faecalis* harvested during the late log phase of growth, just prior to the onset of the stationary phase (*3*). Since the log phase of growth is thought to contain a relatively uniform cell population, in which the rate of protein synthesis is constant and is determined by the ribosomal RNA content (*4*), we investigated the possible alterations in the functional state of messenger RNA in cell-free extracts obtained at various stages of the bacterial growth cycle.

Cells were grown in a phosphate-buffered medium of yeast extract and glucose as described previously (*3*). When cells were to be labeled with P^{32}, the phosphate concentration was decreased from 0.4 to between 0.02 and 0.04 percent, and the medium was supplemented with P^{32} (Squibb, carrier free, 1 μc/ml). Extracts were prepared by the lysozyme procedure (*3*), modified by the addition of 0.5*M* sucrose to the lysing medium. Protoplasts were disrupted by three passages through a large-bore hypodermic syringe. After the extracts had been centrifuged twice at 6000 rev/min to remove intact cells and debris, they were centrifuged at 105,000*g* for 2 hours to give a particulate and a soluble fraction. Fractions containing membranes were obtained from crude extracts sedimented at 30,000*g* for 30 minutes as described previously (*3*). All extracts were fractionated in a buffered medium consisting of $10^{-2}M$ tris-HCl, *p*H 7.6; $10^{-2}M$ magnesium acetate; $10^{-2}M$ β-mercaptoethanol; and 0.9 percent KCl.

Protein synthesis activity in cell-free extracts increases with the age of the culture throughout the log phase of growth and in the subsequent stationary phase (Table 1). The data of Table 1 were obtained with particulate fractions supplemented with supernatant fluid of extracts from cells in late log phase centrifuged at 105,000*g*. If supernatant fluid of extracts from cells in the stationary phase were used, incorporation of amino acids by all extracts was inhibited by over 50 percent.

When extracts of *S. faecalis* are centrifuged in 10 to 35 percent sucrose, three ribosomal fractions are obtained (*3*): (i) ribosomal monomers and subunits (Table 1); (ii) membrane-bound ribosomes containing active polysomes and membrane-bound 50*S* ribosomal subunits; and (iii) aggregates of 30*S* ribosomal subunits held by strands of recently synthesized messenger RNA. The free ribosomes and ribosomal monomers sediment in the upper half of the sucrose gradient; aggregates of 30*S* subunits sediment in a broad peak in the lower half of the gradient. The average sedimentation coefficient of the 30*S* aggregate fraction is 150*S* as estimated with *E. coli* ribosomes as standards. The membrane-bound ribosomes sediment with the pellet and are recovered as such after several washes in solutions containing $10^{-3}M$ Mg^{2+} (*3*).

Throughout the log phase of growth, the content of free ribosomes in the extracts progressively decreases while that of membrane-bound ribosomes increases. During the log phase, the increase in membrane-bound ribosomes parallels the increase of protein synthetic activity in the extracts. This finding agrees with that of a previous re-

port indicating that protein synthesis in centrifugal fractions of *S. faecalis* harvested during the late log phase is a function of the membrane content of the fractions (*3*).

The reason for a marked and continuing increase in the protein synthetic activity of particulate fractions from cells in stationary phase is not clear. However, membrane-bound ribosomes isolated by the sucrose gradient procedure comprise both functional polysomes and membrane-bound 50*S* ribosomal subunits. The latter are inactive in cell-free protein synthesis because the moiety consisting of ribosomal subunit and messenger RNA required for protein synthesis is absent (*3, 5*). The slight, but consistent, decrease in the fraction containing the 30*S* aggregate from cells observed in the stationary phase may be accompanied by an increased number of functional polysomes in the membrane fraction. In extracts of cells from the late log phase, functional polysomes constitute less than 10 percent of the ribosomal content of the membrane fraction (*5*). Consequently, a 3-percent decrease in the 30*S* aggregate fraction could result in doubling the number of functional polysomes in the membrane fraction.

That maximum protein synthetic activity occurs during the stationary phase of growth, when net protein synthesis in intact cells is negligible, seems paradoxical. Studies of an *E. coli* B system have led to essentially the same results (*2*). In intact cells, an optimum rate of protein synthesis may be contingent on a rapid turnover of messenger RNA caused by limitations in the cellular sites available for protein synthesis. Such a high turnover would require both the synthesis and the degradation of messenger RNA. In the cell-free system of *S. faecalis*, synthesis of new messenger RNA is prevented by extensive treatment of extracts with deoxyribonuclease, but its degradation may continue during the preparation of extracts.

When particulate fractions from cells in late log or stationary phase were incubated with supernatants of cells from early log phase centrifuged at 105,000*g*, no release of ribosomes or of ribosomal subunits could be detected after up to 2 hours of incubation at 37°C in the presence of $10^{-2}M$ Mg^{2+}, $6 \times 10^{-2}M$ KCl, and $10^{-2}M$ tris-HCl buffer, *p*H 7.6. However, addition of 10 μmole of inorganic phosphate (sodium or potassium salts) per milliliter caused a release, dependent on time and temperature, of ribosomal constituents from washed membrane fractions. This release occurred both in the presence and in the absence of 105,000*g* supernates, indicating that a particulate polynucleotide phosphorylase is capable of polysome degradation. A polynucleotide phosphorylase has been implicated in the degradation of messenger RNA in uninfected *E. coli*, and in cells infected with bacteriophage (*6*). The enzyme is membrane-bound in *S. faecalis*, strain 9790 (*7*).

The activity of membrane-bound polynucleotide phosphorylase of *S.*

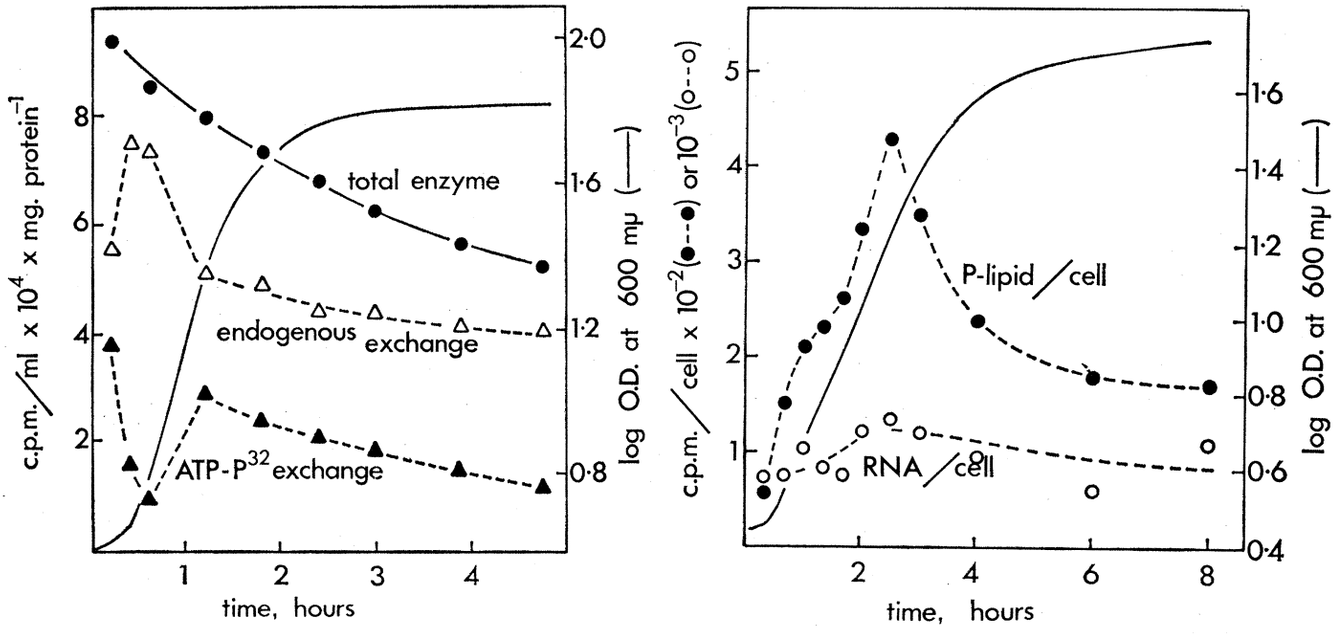

Fig. 1 (left). Alteration in the activity of membrane-associated polynucleotide phosphorylase. Assay by the method of Littauer and Kornberg (*9*) in a 1.0-ml system containing 0.5 ml of a membrane fraction, 0.125 μc of P^{32}, $5 \times 10^{-3}M$ Mg^{2+} and $5 \times 10^{-3}M$ tris-HCl, *p*H 7.6. The mixtures were incubated for 1 hour at 37°C, and 0.2-ml fractions were removed at various times into cold 7 percent perchloric acid. Net incorporation was determined on washed charcoal, collected on Millipore filters. The endogenous exchange reaction was determined in the absence of added nucleotides; the ADP-P^{32} exchange reaction was in the presence of 20 μmole of ADP. The total activity is the sum of the two reactions. An OD of 1.0 corresponds to 0.40 mg (dry weight) per milliliter of the intact cells. Fig. 2 (right). Phospholipid and RNA content per cell. Cells grown in the presence of P^{32} were examined at various times for total phosphatides, after they had been washed three times, precipitated in cold 10 percent trichloroacetic acid (TCA), followed by extraction of the whole cells in a mixture of chloroform and methanol (2:1, by volume). The chloroform phase was washed at least six times with two volumes of 1.5*M* KCl; the pooled washings plus the methanol phase were reprecipitated in 10 percent TCA, collected on Millipore filters, and washed three times with 5 ml of 10 percent TCA. ○--○, Counts per minute per milliliter of culture in the chloroform phase per OD unit at 600 mμ; ●--●, counts per minute per milliliter of culture in the aqueous phase per OD unit at 600 mμ. Under conditions of limited phosphate the log phase is followed by a period of decelerated growth, possibly due to excessive acid production.

faecalis 10Cl is a function of culture age, decreasing with increasing age of the culture (Fig 1). A similar decrease has been reported in the specific activity of the polynucleotide phosphorylase of *Azotobacter vinelandii* and of *Alcaligenes faecalis* (*8*). Enzyme activity was determined from the sum of two reactions (*9*), that is, the exchange of P^{32} into acid-soluble charcoal-absorbable nucelotides in the presence and in the absence of exogenous ADP (Fig. 1). The endogenous reaction may represent membrane-bound enzyme engaged with messenger RNA; the ADP exchange reaction may represent enzyme free from messenger RNA. The precise significance of the alterations in specific activities of the endogenous-P^{32} exchange reaction, and of the ADP-P^{32} exchange reaction remain unclear. The changes are especially striking during the lag phase, just prior to the onset of exponential growth.

Table 1. Activity and ribosomal distribution during culture development.

Age	Growth phase	Optical density at 600 mμ	Incorporation (count/min per mg RNA)*	Free ribosomes	Membrane-bound ribosomes† (% total)	30*S* aggregates
75 minutes	Lag	0.084	45,190	36	46	18
150 minutes	Log	0.300	66,860	19	63	18
210 minutes	Late log	0.770	76,230	12	71	17
11 hours	Stationary	0.740	126,290	9	77	14

* Particulate fractions from various ages were supplemented with 0.2 ml of a 105,000*g* supernate from 210-minute extracts (0.38 mg RNA) and were incubated for 2 hours in a standard 1-ml system (*3*) supplemented with 2.6×10^5 count/min of a mixture of C^{14}-labeled L-amino acids. The assay system contained, per milliliter: ATP, 8 μmole; GTP, 0.4 μmole; magnesium acetate, 10 μmole; β-mercaptoethanol, 8 μmole; creatine phosphokinase, 22 μg; and creatine phosphate, 60 μmole, in 0.04*M* tris-HCl buffer, *p*H 7.6. The reaction products were determined after hot perchloric acid hydrolysis, followed by NaOH solubilization, and by reprecipitation in cold trichloroacetic acid. The incubation mixtures from the various extracts contained 60.2, 63.0, 81.0, and 93.4 μg particulate RNA per milliliter, respectively. † Free ribosomes and 30*S* aggregates were estimated from areas under peaks of OD at 260 mμ obtained after centrifugation in 10 to 35 percent sucrose (in tris-HCl $10^{-2}M$, *p*H 7.6; Mg^{2+} $10^{-3}M$) at 32,500 rev/min for 3 hours in an SW 39 Spinco rotor (*3*). Membrane-bound ribosomes were estimated from the OD at 260 mμ of dilutions of gradient pellets. Control studies using P^{32}-labeled extracts indicate that the optical density obtained under these conditions is due to ribosomal RNA, and not to light scattering (*5*).

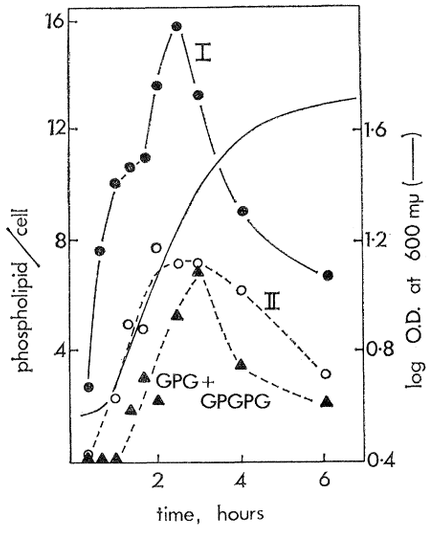

Fig. 3. Membrane content of various phosphatides. Cells were extracted with 20 ml per gram (wet weight) of $CHCl_3$-CH_3OH (2:1, by volume) overnight, filtered through glass wool, and the $CHCl_3$ layer was washed twice with 2*M* KCl and once with distilled water. The $CHCl_3$ was then evaporated to dryness under N_2 at 40°C. Ascending paper chromatography used Whatman SG-81, silicic-acid impregnated paper with a mixture of diisobutyl ketone, glacial acetic acid, and water (40:20:3, by volume). Chromatograms were exposed to DuPont x-ray film for 24 days. The radioautograms were developed and scanned with a Beckman/Spinco Analytrol. Areas under the peaks were determined by the use of planimeter. Four major peaks were observed and tentatively identified as: *I*, alanine ester of phosphatidyl glycerol; *II*, lysine ester of phosphatidyl glycerol; *GPG*, phosphatidyl glycerol; and *GPGPG*, diphosphatidyl glycerol.

Aging cultures of *S. faecalis* and of *E. coli* undergo marked changes in the phospholipid composition of the membrane. There is an increase in ninhydrin-positive phosphatides in the membranes (*10*). In *S. faecalis* 10Cl, attachment of the 50*S* ribosomal subunit to the membrane is susceptible to the action of phospholipase C (*5*), an enzyme capable of hydrolyzing the *O*-amino acid esters of phosphatidyl glycerol (*11*).

Extracts of *S. faecalis* from various stages of growth were analyzed for RNA and phospholipids, and also for the relative concentration of individual phosphatides (*11*). Throughout the log phase of growth there was a continuous increase in the concentration of phospholipid per cell, amounting to an eight-fold rise (Fig. 2). Since the phospholipids of *S. faecalis* are restricted to the cell membrane (*10*), the membrane content per cell must also increase with age. The decreasing ratio of membrane to cell observed after termination of the log phase might be accelerated by a limiting phosphate in the medium. However, it is noteworthy that in the same series of experiments, the ratio of RNA per cell varied by a factor of less than 2.

The membrane content of individual phosphatides varies in a complex way during the log and in the stationary phases of growth (Fig. 3). Of the various phosphatides examined, a ninhydrin-positive compound tentatively identified as an alanine ester of phosphatidyl glycerol (*11*), with an R_F of 0.25, appears to be the principal constituent responsible for the progressive increase in membrane phospholipids per cell. However, the concentration per cell of other phosphatides, the ninhydrin-negative constituents representing phosphatidyl glycerol and cardiolipin, and a ninhydrin-positive component thought to be the lysine ester of phosphatidyl glycerol, increases at a later time than does that of the alanine ester. Results of labeling with C^{14}-amino acid indicate that the lysine ester is the phospholipid associated with the membrane-bound ribosomes in *S. faecalis* (*5*).

Our findings demonstrate that the log phase of growth in batch bacterial cultures is a period of continuous cellular modifications; there is a progressive increase in the net content of functional messenger RNA in cell-free extracts. Since in log cultures of *S. faecalis* the concentration of protein is proportional to the turbidity (*8*), the increasing concentration of messenger in the cell-free extracts must be ascribed to a change in the stability of messenger during the preparation of extracts.

The specific activity of a membrane-bound polynucleotide phosphorylase decreases with aging of the culture. This suggests that with increasing culture age, fewer enzyme units capable of degrading polysomes are present per unit membrane. Messenger RNA is thought to be stabilized at membrane-bound polysomes (*13*), and a progressive increase of these structures was observed as the culture aged. In view of the increase in membrane content per cell, and in particular the increase of the lysine ester of phosphatidyl glycerol, the data suggest the possibility of in-

creased sites for polysome attachment per unit membrane. The two changes under consideration occur at the membrane, progressively throughout the log phase of growth. Consequently, our findings suggest that membrane constituents may play a regulatory role during bacterial growth, possibly through alterations in the number of sites available for polysome attachment, and for the localization of enzymes responsible for messenger degradation.

The range of changes observed in membrane content, and in the extent of ribosomal attachment to membranes during culture aging, might explain some of the negative results obtained on electron microscopic examination of bacterial cultures from early log phase for membrane-bound polysomes (*14*).

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Newberyite in Ancient and Modern Urinary Calculi: Identification and Space Group

Abstract. *Relatively large amounts of newberyite, $MgHPO_4 \cdot 3H_2O$, are found in old or very large urinary calculi. Single crystals of struvite, $MgNH_4PO_4 \cdot 6H_2O$, sometimes show some decomposition to newberyite on aging. The fact that large amounts of struvite are also found in ancient stones implies, however, that special conditions, as yet unknown, are required for decay to occur.*

In 1956 (*1*) newberyite was first identified as a crystalline component of a kidney stone, the other main component being hydroxyl apatite. In 1962 (*2*) it was recorded as a very minor constituent of 250 stones from Leeds, England, and T. Rokkones has found it in one out of 50 Indian and in five out of 25 Indonesian bladder stones studied in Norway (*3*). On the other hand Lagergren (*4*) does not mention it in his studies of bladder and kidney stones from Sweden, nor does Prien (*5*), although the crystallographic studies he made extended over a period of 23 years and covered 25,000 urinary calculi obtained from the United States.

Table 1. Interplanar spacings and the relative intensities of strong and medium reflections in naturally occurring newberyite. S, strong; M, medium; V, very.

hkl	*d*(Å)	*I*	
111	5.941	V S	
020	5.340	S	
200	5.109	M	
021	4.711	V S	
210	4.609	S	
102	4.496	S	
112	4.145	S	
220	3.691	M S	
022	3.652	M S	
202	3.574	M S	
221	3.465	S	*
122	3.437	S	*
131	3.187	M S	
311	3.086	V S	
113	3.043	V S	
023	2.832	M S	†
302	2.816	S	
231	2.805	S	†
132	2.789	S	†
312	2.723	S	
213	2.703	M	
040	2.672	M	
041	2.578	S	†
400	2.555	S	†

* Combine to give one V S line; † Combine to give one S line.

We have found newberyite in 17 percent of the urinary calculi so far analyzed by x-ray diffraction sampling techniques. For identification purposes, the spacings and intensities of the reflections for the naturally-occurring material (*6*) were determined, since different samples of commercial preparations gave slightly different x-ray powder patterns all of which contained strong lines not listed in the ASTM Index (*7*). In the natural deposit a small amount of Mg^{2+} is replaced by Fe^{2+} and Mn^{2+}. Analysis figures (*8*) give the total amount of FeO and MnO as 1 percent. The effect on the unit-cell dimensions is negligible.

Accurate unit-cell dimensions were obtained from single-crystal oscillation photographs taken with the Ievinš-Straumanis mounting. The space group

Table 2. Occurrence of newberyite, $MgHPO_4 \cdot 3H_2O$, in collections of bladder and kidney stones examined by x-ray diffraction methods, compared with occurrence of struvite, $MgNH_4 \cdot PO_4 \cdot 6H_2O$. (a) Number of stones examined; (b) number containing newberyite; (c) number containing struvite; (d) number containing both; (e) percentage of newberyite estimated by sampling; (f) the same for struvite. All analyses were carried out at University College, London.

	Site	Date	(a)	(b)	(c)	(d)	(e)	(f)
			Norwich, England (10)					
Juvenile	Bladder	1773–1909	50	19	12	12	8.6	2.9
Adult	Bladder	1773–1909	33	7	2	2	6.1	1.2
			London, England (11)					
	Bladder	1829–47	8	1	1	1		
			Norwich, England (12)					
(Mostly adult)	Bladder	1932–61	57	2	33	2	0.4	32.7
			Indonesia (13)					
	Bladder	Modern	40	23	24	18	13.4	17.9
			Northeast Hhailand (14)					
Juvenile	Bladder	Modern	58	0	4	0	0	0.7
Adult	Bladder	Modern	19	0	3	0	0	9.9
Adult	Kidney	Modern	63	5	15	4	1.8	17.4
			Turkey (15)					
Juvenile	Bladder	1963–66	50	0	11	0	0	8.1
Juvenile	Kidney	1963–66	91	0	16	0	0	10.5