p-nitrophenyl phosphate; and the membrane phosphatase activity which is sensitive to ouabain requires the internal presence of its substrate (*3*). Also, reconstituted ghosts (*4*), even when red cells are hemolyzed in 500 volumes of hypotonic solution, retain enough of the intracellular phosphatase to mask any contribution from the membrane phosphatase (*3*). We prepared reconstituted ghosts containing very low amounts of the intracellular phosphatase by hemolyzing the cells at 0°C in 300 volumes of hypotonic solution and allowing the hemolyzate to stand at this temperature for 10 minutes (*5*). This procedure allows the intracellular phosphatase activity to almost reach equilibrium with the hemolyzing solution.

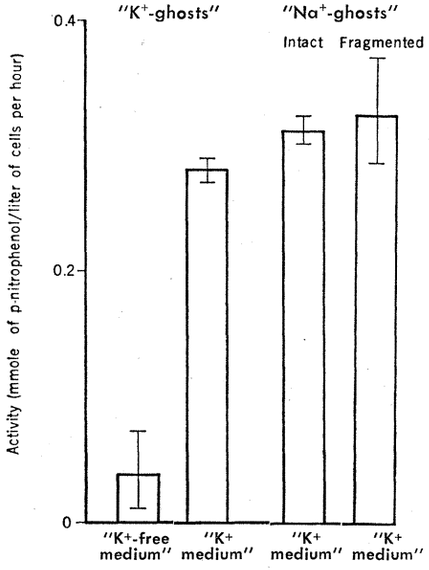

Fig. 1. Ouabain-sensitive hydrolysis (difference between rate of hydrolysis in absence and presence of $10^{-3}M$ ouabain) of *p*-nitrophenyl phosphate in reconstituted ghosts of red cells and in ghosts fragmented by freezing and thawing. The composition of the "K+-ghosts" before sealing was (mmole/liter): KCl, 50; $MgCl_2$, 5; NaCl, 10; and tris(hydroxymethyl)aminomethane (tris) hydrochloride, 85 (*p*H 7.8). The composition of the "Na+-ghosts" was, before sealing (mmole/liter): NaCl, 110; $MgCl_2$, 5; and tris-HCl, 40 (*p*H 7.8). The "K+-medium" contained: in the "K+-ghosts" experiments (mmole/liter), KCl, 100; NaCl, 30; $MgCl_2$, 10; and tris-HCl, 20 (*p*H 7.8); and in the "Na+-ghosts" experiments (mmole/liter), KCl, 50; choline-Cl, 90; $MgCl_2$, 10; and tris-HCl, 10 (*p*H 7.8). The "K+-free medium" was similar to the "K+-medium" except that Na^+ replaced all the K^+. The *p*-nitrophenyl phosphate (6.3 mmole/liter) was present as substrate. The ghosts suspensions were incubated at 37°C for 30 minutes, and the reaction was then stopped by cooling and addition of trichloroacetic acid (TCA) (final concentration, 5 percent, weight to volume). Hydrolysis of *p*-nitrophenyl phosphate was estimated by measuring the concentration of nitrophenol (absorption at 410 nm after removal of TCA-insoluble fraction and adjustment of *p*H to 10 with NaOH). The number of ghosts in the final incubation media was adjusted to give a hematocrit reading of about 5 percent in terms of original cells. In all experiments, not less than 60 percent of the ghosts recovered their low permeability to cations. The vertical lines represent the range of three experiments.

Ghosts were prepared (Fig. 1, composition shown in the legend) and, after "sealing" (*6*) and washing, were incubated in isotonic Na^+ or K^+ media with and without $10^{-3}M$ ouabain. The ouabain-sensitive phosphatase activity (*7*) of ghosts rich in K^+ is largely abolished when the ghosts are transferred from a medium containing K^+ to a medium almost free of K^+. Although this result shows that external K^+ is necessary for full activation, it does not rule out the possibility that K^+ is required at both sides of the membrane. To test this point, reconstituted ghosts with no K^+ inside ("Na^+-ghosts") were assayed for ouabain-sensitive phosphatase activity. A large ouabain-sensitive activity can be detected when K^+-free ghosts are suspended in a K^+-containing medium for a length of time that would not lead to a significant intracellular K^+ accumulation (Fig. 1). The lack of effect of intracellular K^+ is further shown by the fact that the activity of the same ghosts remains unchanged after freezing and thawing, although K^+ should now have access to both sides of the membrane because of disruption of the permeability barrier.

Asymmetrical activation of the membrane phosphatase by external K^+, together with the finding (*3*) that the substrate has to be present internally, suggests that the enzyme system responsible for the K^+-activated hydrolysis of *p*-nitrophenyl phosphate has a definite orientation within the membrane. Presumably as a result of this orientation, the substrate can only reach the active site from the internal surface of the cell membrane, whereas the activator can only have access to its specific site from the external surface of the cell membrane. The similitude of these asymmetrical requirements with those of the $(Na^+ + K^+)$-adenosine triphosphatase system (*2*) is consistent with the proposed relation between both enzymic activities.

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6. "Sealing" means the restoration after hemolysis of the low normal permeability of the red cell membrane to cations after hemolysis. It is accomplished by addition to the hemolyzate of enough concentrated salt solution to restore the tonicity to 315 ideal milliosmols per liter, followed by incubation at 37°C for 40 minutes.
7. Ouabain, having no action in the absence of K^+, selectively abolishes the effect of this cation. For this reason, ouabain-sensitive activity can be taken as synonymous with K^+-dependent activity.
8. Supported by grants from the Consejo Nacional de Investigaciones Científicas y Técnicas (C.N.I.C.T.), Argentina, and the Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires. A.F.R. and P.J.G. are Established Investigators of the C.N.I.C.T.

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Kasugamycin Resistance: 30S Ribosomal Mutation with an Unusual Location on the Escherichia coli Chromosome

Abstract. *A mutation in* Escherichia coli *to resistance to the aminoglycoside antibiotic kasugamycin alters the 30S ribosomal subunit. Though all other known 30S ribosomal mutations are located in a cluster in the streptomycin region, kasugamycin resistance is located at a distance from this region, near the leucine region.*

Mutations to resistance to various antibiotics have proved to be valuable tools for studying the assembly of ribosomes. This communication reports a new locus (*ksg*) affecting the 30*S* ribosomal subunit of *Escherichia coli*, which is unusual in being located on the chromosome at a distance from the known cluster of 30*S* loci in the streptomycin (*str*)-spectinomycin (*spc*) region (*1*, *2*).

Kasugamycin, an antibiotic formed by *Streptomyces kasugiensis*, is widely used

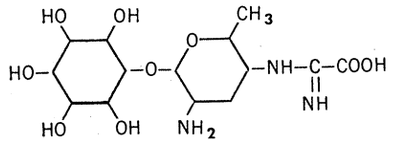

Fig. 1. Structure of kasugamycin.

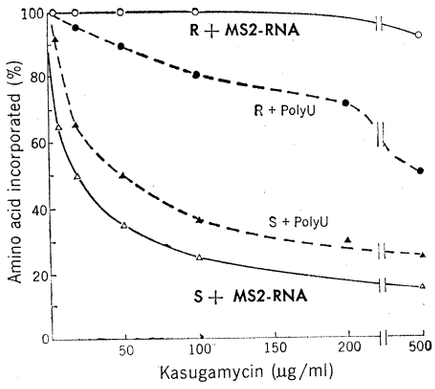

Fig. 2. Inhibition by kasugamycin of ^{14}C-phenylalanine incorporation directed by polyuridylic acid, or ^{14}C-valine incorporation directed by phage MS2-RNA. S is the S30 extract prepared from a Ksg^{s} strain (JS12), R is the S30 extract prepared from a Ksg^{R} strain (FS54). Final Mg^{++} concentration was 10 m*M* (for polyU) or 8 m*M* (MS2-RNA). Other conditions as described (*14*).

in Japan to inhibit *Piricularia oryzae*, the fungus which causes rice blast disease (*3*). This antibiotic consists of D-inositol linked to kasugamine (*4*) (Fig. 1); it is classified as an aminoglycoside, though it lacks the streptamine moiety common to all other aminoglycosides except spectinomycin (*5*). Also, like spectomycin (*6*), kasugamycin fails to cause misreading of the genetic code in vitro (*7*), and it is bacteristatic rather than bactericidal (*8*). Kasugamycin inhibits protein synthesis in vitro, requiring considerably higher concentrations with *E. coli* ribosomes than with *P. oryzae* ribosomes (*9*). The drug apparently acts by inhibiting binding of aminoacyl-tRNA to ribosomes (*10*).

The kasugamycin-resistant (Ksg^{R}) mutants of *E. coli* K-12 strain JC12 were readily obtained by nitrosoguanidine mutagenesis (*11*). Seven mutants were selected on plates containing 1000 μg of kasugamycin per milliliter. They were resistant at concentrations of 1000 μg/ml to > 5000 μg/ml Ksg, whereas the parent strain is inhibited by 100 μg/ml (*12*). A mutant resistant to 5000 μg/ml (FS50 *ksg-3*), and a spontaneous streptomycin-resistant (Str^{R}) derivative (FS54), were used for most of the experiments; they and the other strains employed are listed in Table 1. All Ksg^{R} strains were fully sensitive to streptomycin and spectomycin, and Str^{R} and spectomycin resistant (Spc^{R}) strains were sensitive to kasugamycin. Among seven strains selected for kasugamycin resistance none were dependent.

In polypeptide synthesis in vitro, with either polyuridylic acid (polyU) or bacteriophage MS2-RNA as messenger, the inhibition of ribosomes from a sensitive strain increased with the concentration over a broad range, and maximum inhibition (75 to 85 percent) required over 100 μg/ml (Fig. 2). This behavior contrasts with that of spectomycin and streptomycin, which reach their full effect at 2 to 5 μg/ml with either messenger (*6*). Moreover, the inhibition was less complete with polyuridylic acid than with phage RNA, though the difference was less than that observed with spectomycin or with streptomycin (*6*). Figure 2 further shows that with MS2-RNA as messenger the ribosomes from Ksg^{R} strain were essentially completely resistant to inhibition by kasugamycin, up to at least 500 μg/ml; with polyU, resistance was less complete.

The site of action of kasugamycin was further localized by tests with hybrid ribosomes constructed by exchanging 30*S* and 50*S* subunits from Ksg^{S} and Ksg^{R} strains (Table 2). Sensitivity or resistance to kasugamycin is a property of the 30*S* subunit, just as previously demonstrated for streptomycin (*13*) and spectromycin (*5*). Similar results were obtained with a second preparation of ribosomal subunits.

Mapping both by conjugation and transduction (*14*) was used to test the linkage of *ksg* to the *str-spc* cluster of genes, which affect the 30*S* ribosomal subunit and lie between *argG* and *malA* (*1*). The Hfr strain FS54 (*str ksg-3 purC argG*+ *malA*+) was mated with F− strain FS32 (*str*+ *ksg*+ *purC*+ *argG malA*), and Str^{R} Pur+ recombinants were selected. Of these recombinants 40 out of 100 had the donor alleles of both *argG*+ and *malA*+, but none (0/100) had also received the donor *ksg-3* allele. Moreover, no cotransduction of *str* and *ksg* could be demonstrated with phage P1Kc. It was concluded that *ksg* is not close to the *str-spc* gene cluster.

The location of *ksg-3* was then sought by analysis of linkage to known markers. A more direct approach would be

Table 1. Strains of *E. coli* K-12 used.

Strains	Source	Description
HfrH	D. H. Fraenkel	Hfr; *lac*+
JC12	P. Anderson	Hfr; *met lac purC*
FS50	Nitrosoguanidine mutagenesis of JC12	As JC12, but *ksg-3*
FS54	Spontaneous mutant of FS50	As FS50, but *str-25*
JC411	W. K. Maas	F−; *argG malA met leu lac his str*
FS32	Reference (*14*)	As JC411, but *leu*+ *str*+ *spc*
FS40	FS54 × JC411	As JC411, but *his*+ *ksg-3*

selection of Ksg^{R} recombinants, but there were unexplained difficulties with this selection. A *ksg*+ *argG malA leu his purC*+ recipient (JC411) was mated with *ksg-3* donor FS54, which injects markers clockwise in the order *argG*+ . . . *malA*+ . . . *leu*+ . . . *his*+ . . . *purC*. Arg+ Pur+, Leu+ Pur+, and His+ Pur+ recombinants were selected and were scored for Ksg^{R} (Table 3). Very few recombinants selected for Arg+ were Ksg^{R}, in harmony with the results of the previous cross. However, 43 percent of those selected for His+ were

Table 2. Effect of kasugamycin (Ksg) (100 μg/ml) on polyuridylic acid–directed incorporation of ^{14}C-phenylalanine by hybrid ribosomes. Ribosomes were dissociated into subunits by dialyzing an S30 extract for 4 hours against buffer containing 0.1 m*M* Mg^{++}, 10 m*M* tris-HCl, 60 m*M* KCl, and 6 m*M* mercaptoethanol; subunits were separated by centrifugation in a Spinco SW41 rotor through 14-ml linear sucrose gradients (5 to 20 percent) containing the same buffer without mercaptoethanol. Subunits were brought to 10 m*M* Mg^{++} and were concentrated by ultrafiltration. In order to promote recombination of subunits into hybrid ribosomes, they were incubated at 0°C overnight in 10 m*M* Mg^{++}, 60 m*M* KCl, 10 m*M* tris-HCl, 6 m*M* mercaptoethanol. and 1.0 m*M* spermidine. Each 0.5 ml of reaction mixture contained 0.9 optical density units (260 nm) of ribosomes and 0.15 μl of S100 supernatant from a Ksg^{s} strain. Final concentrations were Mg^{++}, 15 m*M*; spermidine, 0.2 m*M*; polyuridylic acid, 100 μg/ml; other conditions as described (*14*). S, sensitive ribosomes or their subunits from Ksg^{s} strain JC411; R, resistant ribosomes or their subunits from Ksg^{R} strain FS54; S70, undissociated ribosomes from strain JC411; R70, undissociated ribosomes from strain FS54.

Ribosomes		^{14}C-Phenylalanine incorporation (count/min)		Inhibition (%)
30*S* subunit	50*S* subunit	−Ksg	+Ksg	
S	S	2534	1479	42
S	R	1578	964	39
R	S	1289	1187	8
R	R	733	654	11
S70		2632	1320	50
R70		1862	1731	7

Table 3. Linkage of *ksg* in conjugation. The donor and recipient strains are listed separately to aid in interpretation of the data. Matings were interrupted at 60 minutes in the selections for ArgG$^+$ PurC$^+$ and Leu$^+$ PurC$^+$, and at 120 minutes for His$^+$ PurC$^+$. Recombinants were purified once before being placed on appropriate mediums for scoring the phenotype.

Donor FS54	*argG*$^+$	*str*	*malA*$^+$	*leu*$^+$	(*ksg-3*)	*his*$^+$	*purC*
Recipient JC411	*argG*	*str*	*malA*	*leu*	(*ksg*$^+$)	*his*	*purC*$^+$

Experimental results

Phenotype selected	Scored (No.)	Ksg^R (No.)	Ksg^R (%)	Comment
ArgG$^+$ PurC$^+$	120	6	5	4 Leu$^+$; 4 Ksg^R; 0 Ksg^S
Leu$^+$ PurC$^+$	120	86	72	
His$^+$ PurC$^+$	178	78	43	60 Leu$^+$; 50 Ksg^R; 10 Ksg^S 118 Leu$^-$; 28 Ksg^R; 90 Ksg^S

Ksg^R, compared with 72 percent of those selected for Leu+. Since *his* is distal to *leu*, these data show that *ksg* is closer to *leu* than to *his*. Moreover, the recombinants selected for His+ were more often Ksg^R (78/178) than Leu+ (60/178); hence *ksg* appears to lie between *leu* and *his* (that is, clockwise to *leu*). Finally, in this cross the proposed sequence *leu-ksg-his* would require two recombinational exchanges to produce Leu− Ksg^R His+ and four exchanges to produce Leu+ Ksg^S His+; whereas the crossover requirements would be reversed if the sequence were *ksg-leu-his*. The proposed sequence is therefore further supported by the finding that the 178 His+ recombinants included 28 Leu− Ksg^R but only 10 Leu+ Ksg^S (Table 3).

The location of *ksg-3* was confirmed by a cross with HfrH, which donates clockwise with *leu* as its earliest marker and *lac* 10 minutes later. This *leu*+ *ksg*+ *lac*+ *str*+ strain was mated for 30 minutes with a *leu ksg-3 lac str* recipient (FS40). Of 60 Leu+ Str^R recombinants, 40 had received the donor Ksg^S marker, but only 2 had also received the donor Lac+ marker. Thus, *ksg-3* lies closer to *leu* than to *lac*.

Though further experiments are required to map the location of *ksg-3* precisely, it is clear that mutations which affect the 30*S* subunit do not all map in a single cluster. This finding may be important for our understanding of the control of the synthesis of 30*S* ribosomal components.

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Regenerative Calcium Release within Muscle Cells

Abstract. *Free calcium appears to trigger the release of stored calcium from the sarcoplasmic reticulum of skinned skeletal muscle fibers immersed in solutions with a low concentration of magnesium ion.*

There is considerable evidence that skeletal muscle myofilaments are activated by calcium (*1*) which is released into the surrounding space by the sarcoplasmic reticulum (*2*). The process is initiated physiologically by a decrease in the electrical potential across the surface membrane (*3*), but the mechanism of this release is poorly understood. The experiments reported here indicate that the release process has the capacity to be regenerative. This conclusion is based on an analysis of the contractions induced when fibers without their outer membranes are immersed in solutions of varied composition.

Single fibers from the semitendinosus muscle of the frog *Rana pipiens* were isolated in silicone oil (*4*), and the surface membrane was removed (*5*) to allow externally applied solutions free access to the myofilament space. Segments of these "skinned" fibers (1 to 3 mm long) were mounted in a force-measuring apparatus (*6*) before they were immersed in aqueous solutions (*7*). Low concentrations of calcium ion in the medium were controlled with ethylene glycol bis(aminoethylether)-*N*,*N*′-tetraacetic acid (EGTA), a calcium chelator with relatively little affinity for magnesium (*8*). Low concentrations of magnesium ion were established when the concentration of the metal was less than that of adenosine triphosphate (ATP) with which it forms a complex (*8*).

Skinned fibers immersed in solutions containing calcium buffered with EGTA slowly accumulate calcium for many seconds before developing force (*9*), and they retain this calcium when transferred to solutions of low concentrations of EGTA. Segments could therefore be loaded with calcium by immersion in a buffered calcium solution and then rinsed free of buffer in EGTA solutions. After this preparation, fibers that were exposed to unbuffered, "free" calcium solutions produced a quick contraction which was superimposed on a much slower contraction (Fig. 1A). The quick contraction could be interrupted by transferring the fiber to a solution containing a high concentration of EGTA (Fig. 1B); it did not depend on the major anion of the bathing medium, as it occurred equally well in solutions of potassium propionate and potassium chloride. The respones required that the fibers be loaded in a buffered calcium solution and that the concentration of free magnesium be relatively low. When fibers were immersed directly in the free calcium without exposure to the buffered calcium (Fig. 1C), or when the concentration of magnesium in solution exceeded that of ATP by 1 m*M*, quick contractions did not occur.

A quick contraction was also in-