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Effect of Anisomycin on the Cellular Level of Native Ribosomal Subunits†

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ABSTRACT: Treatment of Ehrlich ascites cells with anisomycin induces an almost threefold increase in the level of native 60S ribosomal subunits. This increase is not the result of an increase in rate of synthesis or transport of these subunits but is caused by a defect in the joining of the 60S subunit to the smaller initiation complex to form an 80S complex. Experimental evidence for such a blocking of the "joining reaction" could be found in the formation of "half-mer"-type oligosomes and by the release of extra 40S subunits when these oligosomes were treated with ribonuclease. Cycloheximide, an inhibitor of the translocation reaction, and inhibitors of the initiation prevent the increase of native 60S subunits induced by anisomycin. Our results imply that the increase of 60S subunits induced by anisomycin may be helpful in estimating the amount of initiating mRNAs in the cell.

It has been described by several authors that in cultured cells large pools of native ribosomal subunits are present.[1] These subunits may have an important function in the control of initiation of protein synthesis because they serve as a reservoir of initiation factors (Ayuso-Parilla et al., 1973; Freienstein and Blobel, 1975; Sundkvist and Staehelin, 1975; van Venrooij and Janssen, 1976). Under normal growth conditions the level of native subunits is fairly constant. It has been found that limitation of protein synthesis (with NaF, cycloheximide, and deprival of amino acids or glucose) does not influence the total amount of native ribosomal subunits significantly (Hirsch et al., 1973). In this paper we report the effect of anisomycin, an antibiotic isolated from cultures of various streptomyces (Sobin and Tanner, 1954), on the level of native ribosomal subunits. Anisomycin (2-*p*-methoxyphenylmethyl-3-acetoxy-4-hydroxylpyrrolidine; Beereboom et al., 1965) inhibits protein synthesis in mammalian cells but not in bacteria (Pestka, 1971). It has been shown that the transpeptidation step is reversibly inhibited by the binding of anisomycin to the peptidyltransferase center of the large ribosomal subunit (Barbacid and Vazquez, 1975). In addition to these data we now present evidence that treatment of Ehrlich ascites tumor cells with anisomycin also leads to increased levels of native 60S subunits. This effect is explained by experimental evidence that binding of native 60S subunits to the Met-tRNA$_f$–mRNA–40S initiation complex is inhibited by the antibiotic.

Materials and Methods

Materials. Tissue culture media and new-born calf serum were bought from Flow Lab Ltd. All chemicals used were of analytical grade. [5,6-^{3}H]Uridine (sp. act. 51 Ci mmol^{-1}) was bought from the Radiochemical Centre, Amersham. Anisomycin (a generous gift from Pfizer Inc.) was added to the medium as a 1 mM solution in water. All buffers were standardized to the desired pH with concentrated HCl at 4 °C, and, unless otherwise stated, all operations were carried out at 0–4 °C. Centrifugation gravity values are quoted as the average *g* values generated at particular speeds of rotation.

Cell Culture Conditions. The Ehrlich ascites tumor cells were grown in spinner culture as described earlier (van Venrooij et al., 1970) using MEM-S autopow neutralized with $NaHCO_3$ and complemented with 0.15% lactalbumin hydrolysate, 2 mM glutamine, and 10% new-born calf serum. Penicillin (100 IU mL^{-1}) and streptomycin (50 μg mL^{-1}) were routinely used. Three hours before the start of the experiment the cells were replenished with fresh medium (van Venrooij et al., 1970) and incubated at a density of 2×10^6 cells mL^{-1}.

Cell Fractionation. The cell fractionation procedure has been described in detail (van Venrooij et al., 1975). In short, cells were washed and, after swelling in a hypotonic buffer, homogenized by 5–10 strokes of a tight-fitting Dounce ho-

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[1] Native subunits are defined here as those subunits that appear as free subunits in cell extracts, in contrast to the subunits which are present in ribosomes. We refer to the smaller and larger mammalian subunits as the 40S and 60S subunits, respectively. These designations are for the purpose of identification only and do not imply accurate measurements of sedimentation coefficients.

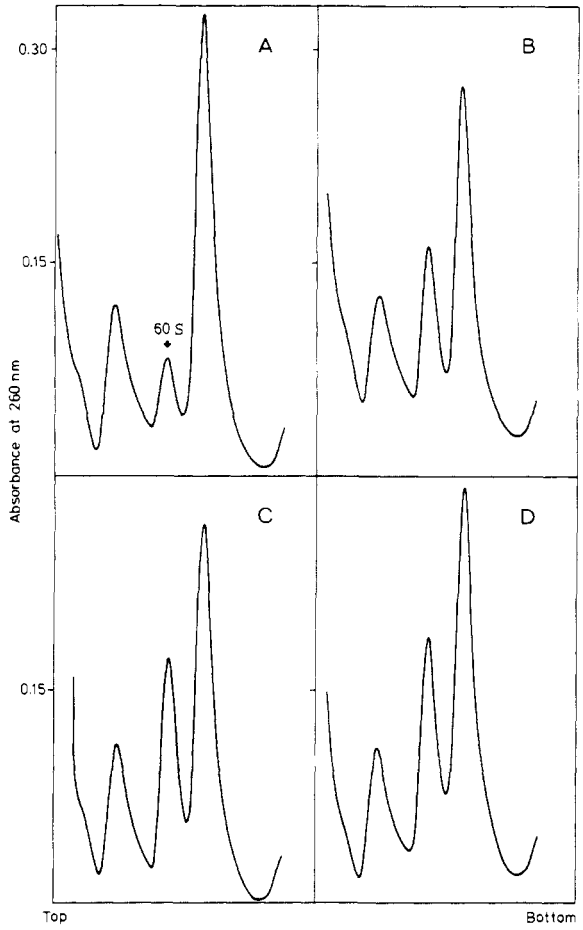

FIGURE 1: Effect of anisomycin on the level of native ribosomal subunits in Ehrlich ascites tumor cells. Two flasks with cells from the same batch were used. One of them, containing 25 mL of cells, served as a control (A). To the 75 mL of cells in the other flask anisomycin (1 μM) was added; 25 mL of anisomycin-treated cells was harvested after 5 min (B), 15 min (C), and 30 min (D), respectively. The control was harvested at timepoint 30. The cells were fractionated on sucrose gradients and the subunit profile was recorded as described under Materials and Methods. Centrifugation was for 4.5 h at 200 000g in an IEC type SB-283 rotor.

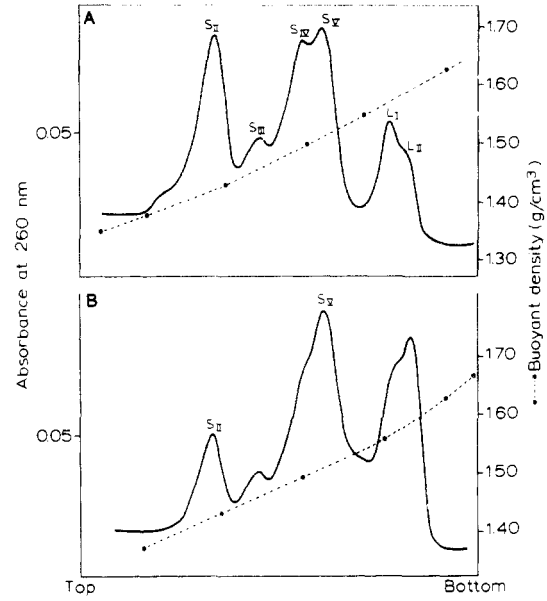

FIGURE 2: CsCl density analysis of native ribosomal subunits isolated from control and anisomycin-treated cells. Native ribosomal subunits from control and anisomycin-treated cells were isolated from sucrose gradients of the type shown in Figure 1. After fixation with 6% formaldehyde the particle suspension was layered onto a preformed CsCl gradient as described under Materials and Methods and centrifuged for 20 h at 147 000g: (A) native ribosomal subunits from control cells; (B) native ribosomal subunits isolated from cells treated with 1 μM anisomycin for 30 min.

mogenizer in buffer 1 (0.05 M KCl, 0.005 M $MgCl_2$, 0.01 M Tris-HCl (pH 7.4), 6 mM 2-mercaptoethanol) containing 0.1% Triton X-100 and 0.25 M sucrose (10^8 cells per 2 mL of homogenization medium). The homogenate was centrifuged for 10 min at 20 000g and the resulting 20 000g supernatant was used as a source of the native subunits.

Sucrose Gradient Centrifugation. About 1 mL of the 20 000g supernatant was layered onto an isokinetic gradient of 15–35% (w/w) sucrose in buffer 2 (50 mM triethanolamine (pH 7.4), 5 mM $MgCl_2$, 25 mM KCl) and centrifuged for 4–6 h at 200 000g in an IEC type SB-283 rotor. Profiles were recorded with a continuous flow monitoring system using a Gilford spectrophotometer. Relative amounts of the various components in the gradients were calculated from the absorbance profiles at 260 nm.

CsCl Gradient Analysis. Fractions of the sucrose gradient containing the native 40S and 60S subunits were pooled and fixed with 0.2 vol of a freshly neutralized 37% formaldehyde solution at 0 °C for at least 10 min. Two milliliters of the fixed particles was layered onto 9 mL of a preformed linear CsCl gradient (density range 1.30–1.75 g/cm^3) in buffer 2 containing 4% formaldehyde, 0.5 mg/mL Nonidet P-40, and 80 μg/mL 2,4-dithioerythritol. The preparations were then centrifuged in a Spinco SW41-Ti rotor at 147 000g and 4 °C for about 20 h. In most experiments the upper 4 mL of the gradient (densities below 1.35 g/cm^3) was discarded and is, therefore, not shown in the figures.

Results

Effect of Anisomycin on the Rate of Protein Synthesis and Cellular Level of Native Subunits. Anisomycin (1 μM) added to a suspension of Ehrlich ascites tumor cells inhibits protein synthesis by more than 90% within a few minutes (not shown). This is in agreement with the many reports describing such an inhibiting action (Pestka, 1971; Vazquez, 1974).

The effect of the antibiotic on the cellular level of native ribosomal subunits is illustrated in Figure 1. While the amount of 40S ribosomal subunits is relatively unchanged, the level of native 60S subunits increases significantly within 5 min after addition of the inhibitor. Under normal conditions, these rapidly growing cells contain about 5 times as much native 40S subunit particles as native 60S subunit particles. This phenomenon of nonstoichiometry of the native subunits seems to be a general one in rapidly growing cells and has been explained recently by the observed different degradation rates of 40S and 60S subunits (Nissen-Meyer and Eikhom, 1976). During anisomycin treatment, however, the ratio native 40S/native 60S subunits changes notably. From the absorbance profiles of 15 experiments we calculated that the 40S/60S particle ratio of 4.8 in untreated cells decreased to 1.7 in anisomycin-treated cells.

Effect of Anisomycin on the Several Classes of Native Subunits Detectable after Isopycnic Gradient Centrifugation. We have recently described that in Ehrlich ascites cells two classes of native 60S subunits can be distinguished using a high-resolution CsCl gradient technique (van Venrooij and Janssen, 1976). One class with a buoyant density of 1.57 g/cm^3, referred to as L_I particles, contains extra nonribosomal protein which can be removed by a high salt wash giving rise

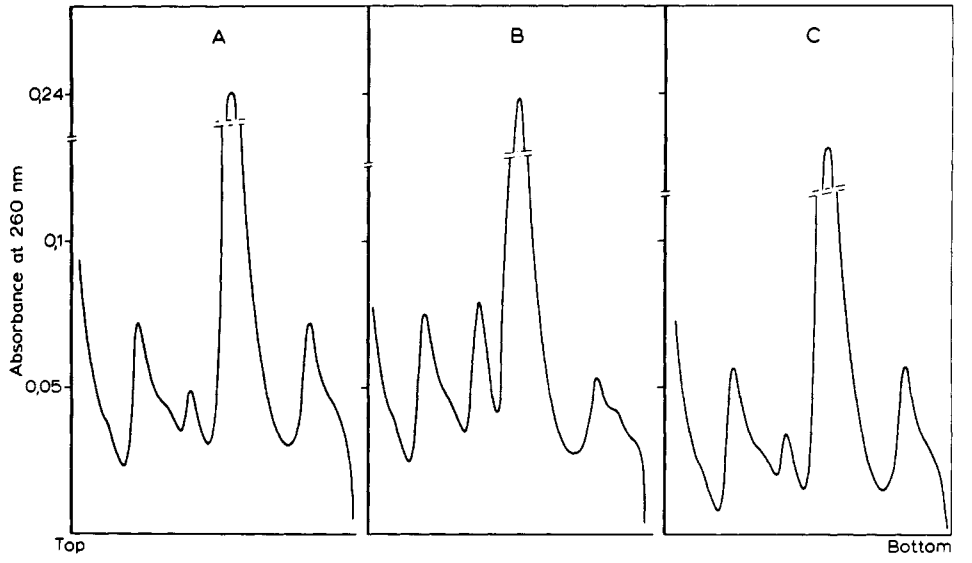

FIGURE 3: Effect of cycloheximide on the increase of native 60S subunits induced by anisomycin. Three hours after replenishment the cells were divided into four groups, A, B, C, and D: (A) control; no additions; (B) anisomycin (1 μM) was added and incubation of the cells was continued for 30 min; (C) cycloheximide (100 μg mL^{-1}) was added; after 10 min anisomycin (1 μM) was added and incubation of the cells was continued for 30 min; (D) as in A, but cycloheximide (100 μg mL^{-1}) was added. The cells were fractionated as described under Materials and Methods. Sucrose gradients were spun for 4 h at 200 000g. The profile of D was identical with the one shown in A and is, therefore, not shown.

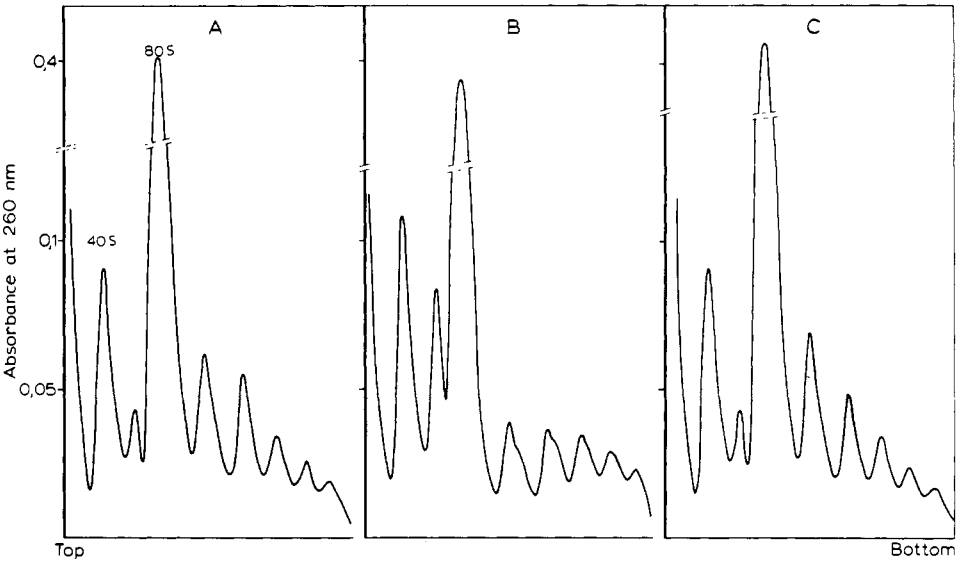

FIGURE 4: Effect of anisomycin on the polyribosomal profile. The experiment was performed as described in the legend to Figure 3. To examine the polyribosomal profiles the sucrose gradients were spun for 2.5 h at 200 000g in an IEC type SB-283 rotor: (A) control, no additions; (B) anisomycin (1 μM) was added and incubation of the cells was continued for 30 min; (C) cycloheximide (100 μg mL^{-1}) was added first; after 10 min anisomycin (1 μM) was added and incubation of the cells was continued for 30 min. The profile of the control treated with cycloheximide (see legend to Figure 3) was identical with the one shown in A.

to particles with the same density (1.59 g/cm^3) as L_{II} particles (van Venrooij and Janssen, 1976). Total native subunits from control and anisomycin-treated cells were pooled from a sucrose gradient of the type shown in Figure 1, fixed with formaldehyde, layered onto a preformed CsCl gradient, and centrifuged for 20 h at 147 000g. Figure 2 shows that both classes of 60S subunits increase.

The small ribosomal subunits in these cells consist of at least five classes of particles. These particles, with buoyant densities of 1.39, 1.42, 1.45, 1.49, and 1.51 g/cm^3, were designated as S_I, S_{II}, S_{III}, S_{IV}, and S_V, respectively (Van Venrooij et al., 1976). Although the total amount of native 40S subunits does not change very much after anisomycin treatment, the CsCl gradients show very reproducibly that S_{II} particles (buoyant density 1.42 g/cm^3) decrease after anisomycin treatment. In all experiments of this type performed we observed a decrease in the amount of S_{II} particles of about 50%.

Does Anisomycin Release 60S Subunits from Cytoplasmic Membranes or from Nuclear Pools? Although anisomycin is primarily known as an inhibitor of protein synthesis, the increase of native 60S subunits could be the result of some side reaction of the drug. Therefore, we tested whether the observed increase in native 60S subunits could be the result of a decreased binding of 60S subunits to cytoplasmic membranes or an increased transport from nuclear pools.

Although the first possibility is not very likely because a relatively small percentage of the native 60S subunits is bound to cytoplasmic membranes, we tested it as follows. Membranes from control and anisomycin-treated cells were isolated and dissolved in a mixture of 0.5% sodium deoxycholate and 0.3%

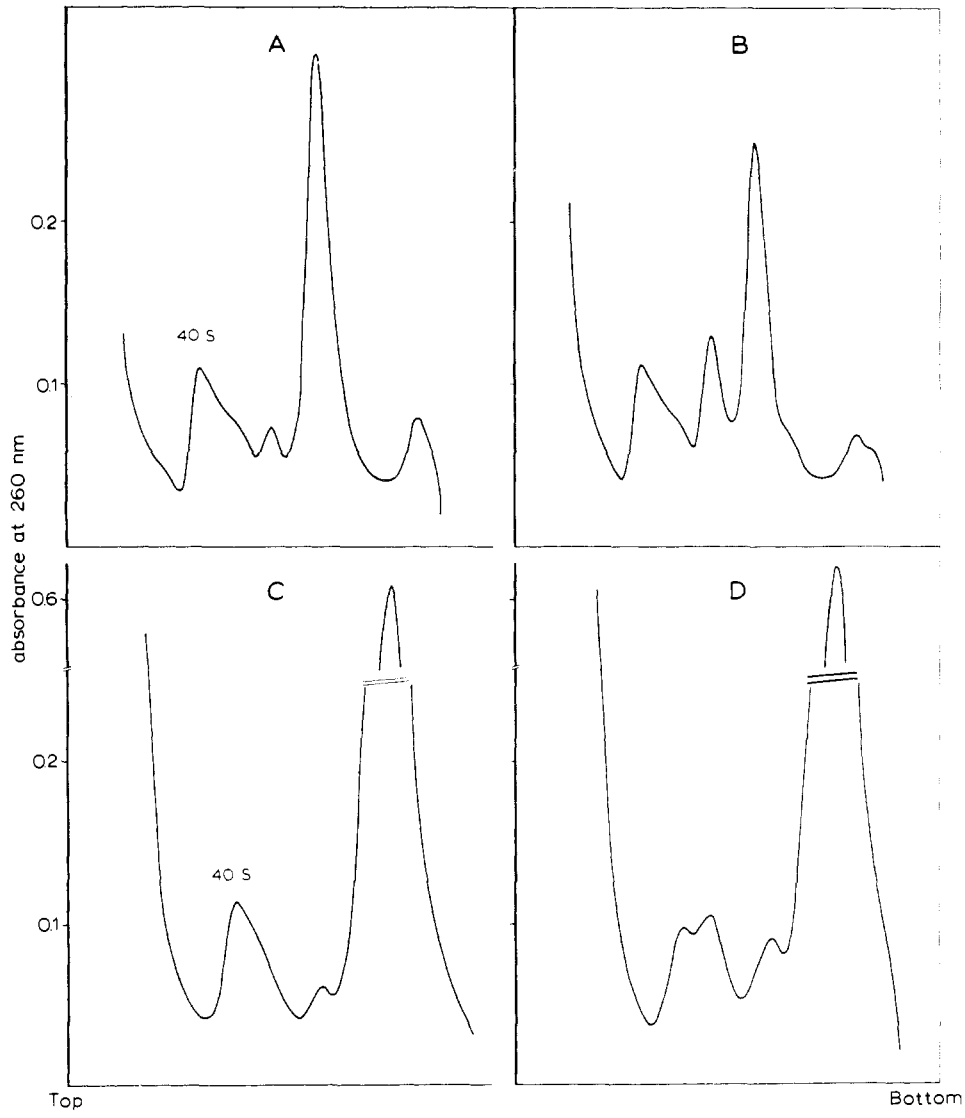

FIGURE 5: Effect of a ribonuclease containing strip gradient on the native subunit profile of control and anisomycin-treated cells; 20 000*g* supernatants of control and anisomycin-treated cells were isolated as described in Materials and Methods. Two types of sucrose gradients were used in this experiment. Isokinetic 15–35% sucrose gradients (see Materials and Methods) were used in A and B. In C and D the upper 2 mL of the 15–35% sucrose gradients was removed and replaced by 1 mL of 15% sucrose in buffer 2 containing 5 μg of RNase A and 1 mL of 10% sucrose in buffer 2. Centrifugation was for 4.5 at 200 000*g* at 10 °C: (A and C) control cells; (B and D) cells treated with 1 μM anisomycin for 30 min.

Triton X-100 (Van Venrooij et al., 1975). After removal of the nuclei by a short centrifugation the supernatants were analyzed by sucrose gradient centrifugation. The profiles obtained showed that the amount of 60S subunits bound to cytoplasmic membranes was essentially unchanged after anisomycin treatment (data not shown). To test the second possibility we labeled ascites cells with [^{3}H]uridine for 1 or 2 h. Then anisomycin was added to part of the cells for 15 min. The results demonstrated that the increase in the amount of 60S subunits induced by the addition of anisomycin is not accompanied by an increased inflow of newly synthesized labeled ribosomal subunits (data not shown).

The Effect of Anisomycin on the Binding of 60S Subunits to 40S Initiation Complexes. The experiments described above strengthened our idea that anisomycin might inhibit one or more steps in the subunit cycle of native 60S subunits. Therefore, we tested whether cycloheximide, an inhibitor of protein synthesis, when added to the cells just before the addition of anisomycin prevents the observed effect of the latter. Figure 3 shows that this is indeed the case. We chose cycloheximide because this drug is primarily an inhibitor of translocation (Barbacid and Vazquez, 1975). Thus, fixing the polysomes by cycloheximide, a process which impedes recycling of subunits released during or after termination, also prevents the observed threefold increase in native 60S subunits. In view of the fact that anisomycin binds to 60S subunits (Barbacid and Vazquez, 1975) a possible site of action could be that binding of native 60S subunits to the 40S initiation complex is prevented by the antibiotic. In that case one would expect the appearance of the so-called "half-mer"-type oligosomes (1.5-mers, 2.5-mers, etc., visualized as split peaks in the sucrose gradient profile) after anisomycin treatment. This phenomenon can indeed be observed as is shown in Figure 4. Consistent with these results is the observation that pretreatment of the cells with cycloheximide, which prevents the increase in the amount of native 60S subunits (Figure 3B), also prevents the appearance of incomplete initiation complexes (Figure 4C).

Ribonuclease Treatment of Incomplete Initiation Complexes. The formation of incomplete initiation complexes during incubation of the cells with anisomycin can be visualized more clearly after treatment of the polyribosomes present in the 20 000*g* supernatant with ribonuclease. Such a treatment

will distinguish between complete (80S) and incomplete (40S) initiation complexes. In the control most of the complexes have a joined 60S subunit. Therefore, ribonuclease cleavage of the polysomes will result in the formation of 80S monoribosomes with little increase in the 40S region of the gradient. When anisomycin is present, however, cleavage of the "half-mer"-type polysomes will also release 40S initiation complexes (Kappen and Goldberg, 1976). We first tried direct treatment of the supernatant with ribonuclease. The results, however, showed a considerable loss of native 40S and 60S subunits. To avoid such an artefactual loss we introduced the ribonuclease in the upper part of the sucrose gradient (see legend to Figure 5). This method proved to be more successful, although part of the native subunits still seems to aggregate giving rise to the formation of, e.g., a 70S component which can be detected in some of the profiles as a shoulder on the light side of the 80S peak. Nevertheless, the results of experiments as depicted in Figure 5 strongly suggest that, only after anisomycin treatment, extra 40S subunits (visualized by the extra peak at about 48 S in Figure 5D) are released by ribonuclease treatment.

Inhibition of Initiation and the Anisomycin Effect. Our results described above suggest that in cells treated with anisomycin part of the mRNAs are able to bind native 40S subunits. The 60S subunits do not join these newly formed initiation complexes and as a consequence the amount of native 60S subunits in the cytoplasm shows an increase relative to the amount of native 40S subunits present. We assumed that this increase of native 60S subunits after anisomycin treatment would not appear when the 40S subunits were also prevented from binding to the available initiation sites. We have tried to test this assumption by adding anisomycin to cells in which rate of initiation of protein synthesis was very low.

NaF is well known as an inhibitor of initiation (see, for example, O'Rourke and Godchaux, 1975) and does allow runoff (elongation) to proceed. Glucose deprival (and the presence of 2-deoxyglucose) lowers the ATP level in the cell and although this results in an inhibition of elongation as well, the decrease in rate of initiation is much more severe. As expected, pretreatment of the cells with 15 mM NaF (Figure 6, B_1 and B_2) or glucose deprival in the presence of deoxyglucose (Figure 6, C_1 and C_2) completely prevented the anisomycin-induced increase of native 60S subunits.

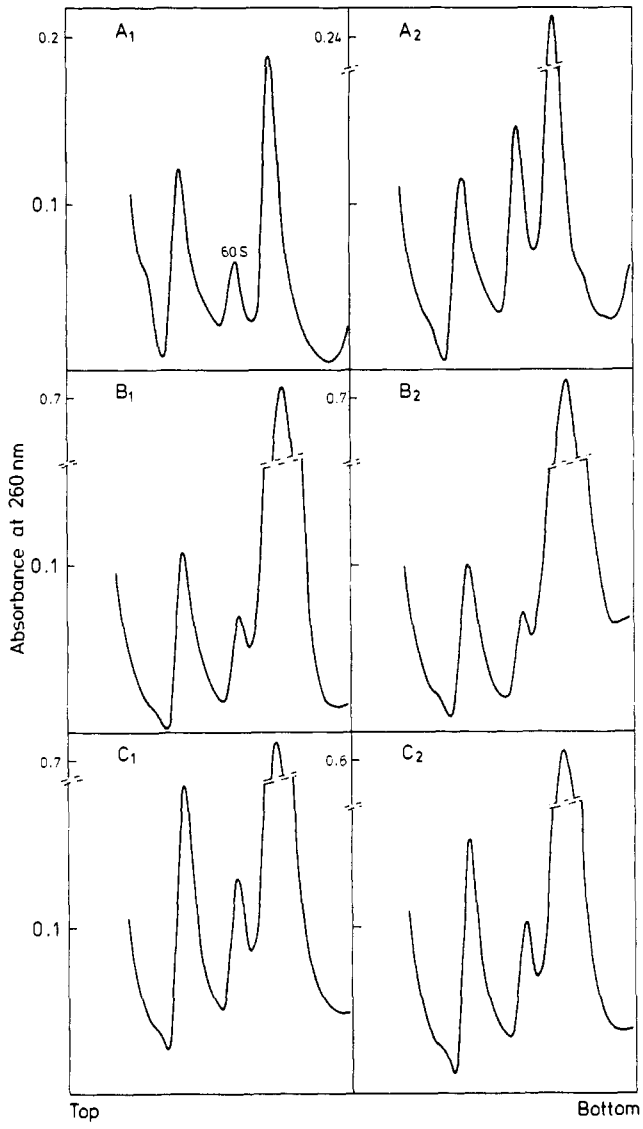

FIGURE 6: The effect of NaF and glucose deprival on the increase of native 60S subunits induced by anisomycin. Three hours after replenishment the cells were divided over 3 flasks, A, B, and C. The cells in flask A were incubated in complete medium for another 60 min. To flask B NaF (15 mM) was added whereafter incubation was continued for 60 min. The cells in flask C were isolated by centrifugation and then resuspended in complete medium without glucose. After 30 min 0.1 mM 2-deoxyglucose was added and incubation continued for another 30 min. After the 1-h incubation half of the cells of flasks A, B, and C (referred to as A_1, B_1, and C_1, respectively) were harvested. To the other half of the cells anisomycin (1 μM) was added. After 30 min, these cells were harvested (A_2, B_2, and C_2, respectively). The cells were homogenized and the homogenate fractionated as described under Materials and Methods. Sucrose gradients were spun for 4.5 h at 200 000*g*.

Discussion

In this paper we have shown that anisomycin increases the intracellular level of native 60S subunits in Ehrlich ascites tumor cells, probably by inhibiting the joining of a 60S subunit with the 40S initiation complex. The most convincing evidence for the formation of incomplete initiation complexes in the presence of anisomycin was obtained by the appearance of "half-mer"-type oligosomes (Figure 4) and their cleavage with ribonuclease (Figure 5). While ribonuclease treatment of a control leads to the formation of 80S monoribosomes, in the presence of anisomycin an extra 40S peak could be observed as well indicating that some 40S subunits bound to the initiation site of some mRNAs in the polyribosomes did not have a 60S subunit attached to them. From similar findings Kappen and Goldberg concluded that the inhibition by pactamycin of polypeptide chain initiation in reticulocyte lysates is also associated with a defect in the "joining reaction" (Kappen et al., 1973; Kappen and Goldberg, 1976).

When 40S subunits leave the subunit pool to bind onto the mRNA one would expect a decrease in the absolute amount of these subunits unless new 40S subunits are generated from monomer dissociation or the last termination reaction. The finding that we did not observe a significant decrease in the total amount of native 40S subunits after anisomycin treatment indicates that binding of a 40S subunit onto the mRNA reactivates one or more protein factors which are able to generate a new 40S subunit and necessarily a new 60S subunit as well. Thus, it seems likely that such a factor, which has been shown to be present on native subunits (Lubsen and Davies, 1972, 1974), may be released when the small subunit binds to the initiation site of the mRNA. From the CsCl profiles (Figure 2) it can be seen that the amount of S_{II} particles (buoyant density 1.42 g/cm^3) decreases after anisomycin treatment. It has been shown earlier that Met-tRNA$_f$ is bound to S_{II} particles (Pain and Henshaw, 1975; van Venrooij et al.,

1976) and the fact that iodinated globin mRNA binds to this class of 40S particles in a cell-free system (W. J. van Venrooij, unpublished results) corroborates the assumption that S_{II} particles did bind to the mRNA and were replaced in the subunit pool by 40S particles with a higher buoyant density (i.e., with less accessory protein). It is now clear that the increase in the amount of native 60S subunits is the net result of two reactions. The 60S subunits do not leave the pool because of the action of anisomycin and new 60S subunits are flowing into the pool as a result of the reactions just described. This mechanism implies that the number of new 60S subunits after anisomycin treatment is equal to the number of initiation complexes in the cytoplasm. This means that the latter number, an important parameter in studies on the kinetics of protein synthesis in vivo, can now be determined experimentally. The fact that limitation of initiation prevents the increase of native 60S subunits after anisomycin treatment (Figure 6) is consistent with this conclusion. We also found that cycloheximide prevents the action of anisomycin. This effect might well be explained by the fact that cycloheximide, in the concentration used, inhibits initiation. On the other hand, an inhibition of translocation by cycloheximide would produce similar effects. Posttranscriptional ribosomes (i.e., those with peptidyl-tRNA bound to the P site) have a higher affinity for anisomycin than pretranslocational ribosomes (i.e., those with the peptidyl-tRNA in the A site) (Barbacid and Vazquez, 1975). Fixation of the peptidyl-tRNA in the A site, for example by pretreatment of the cells with cycloheximide, will therefore diminish or even prevent an action of anisomycin, as is illustrated in Figures 3 and 4.

Acknowledgments

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