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The anaerobic ribonucleoside triphosphate reductase from *Escherichia coli* requires *S*-adenosylmethionine as a cofactor

(deoxyribonucleotide synthesis/oxygen sensitivity)

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ABSTRACT **Extracts from anaerobically grown *Escherichia coli* contain an oxygen-sensitive activity that reduces CTP to dCTP in the presence of NADPH, dithiothreitol, Mg^{2+} ions, and ATP, different from the aerobic ribonucleoside diphosphate reductase (2′-deoxyribonucleoside-diphosphate: oxidized-thioredoxin 2′-oxidoreductase, EC 1.17.4.1) present in aerobically grown *E. coli*. After fractionation, the activity required at least five components, two heat-labile protein fractions and several low molecular weight fractions. One protein fraction, suggested to represent the actual ribonucleoside triphosphate reductase was purified extensively and on denaturing gel electrophoresis gave rise to several defined protein bands, all of which were stained by a polyclonal antibody against one of the two subunits (protein B1) of the aerobic reductase but not by monoclonal anti-B1 antibodies. Peptide mapping and sequence analyses revealed partly common structures between two types of protein bands but also suggested the presence of an additional component. Obviously, the preparations are heterogeneous and the structure of the reductase is not yet established. The second, crude protein fraction is believed to contain several ancillary enzymes required for the reaction. One of the low molecular weight components is *S*-adenosylmethionine; a second component is a loosely bound metal. We propose that *S*-adenosylmethionine together with a metal participates in the generation of the radical required for the reduction of carbon 2′ of the ribosyl moiety of CTP.**

Ribonucleotide reductases catalyze the synthesis of the four deoxyribonucleoside triphosphates (dNTPs) required for DNA replication (1–4). The enzymes provide a link between RNA and DNA metabolism. They have attained special biological interest in connection with recent theories concerning the evolution of life on earth since their appearance was a prerequisite for the transition of an "RNA world" into a "DNA world" (5). Several forms of the enzyme occur in nature, with different structures and catalytic mechanisms. Also, their chemistry is of considerable interest. The enzyme from *Escherichia coli* provided the first example of a biological process that uses a protein radical as a mechanistic option (6, 7). While the direct reduction of ribonucleotides via a radical mechanism is a general mode of dNPT synthesis in all organisms, the way in which this is achieved varies. At present, three classes of ribonucleotide reductases have been described: The aerobic *E. coli* enzyme (8) is the prototype for class I enzymes (2′-deoxyribonucleoside-diphosphate:oxidized-thioredoxin 2′-oxidoreductase, EC 1.17.4.1), also present in all higher animal (9) and plant cells (10). These enzymes consist of two proteins: one large homodimer (protein B1 of *E. coli*) and one small homodimer (protein B2). Characteristic features are the presence of a ferric:tyrosyl radical center in B2 (6) and redox-active dithiols in B1 (11), both involved in the catalytic process. B1 also provides the reductase with allosteric properties (12). A second, as yet poorly characterized, microbial reductase (class II) contains manganese in place of iron (13) but may in many respects be similar to class I enzymes. Class III enzymes (2′-deoxyribonucleoside-triphosphate:oxidized-thioredoxin 2′-oxidoreductase, EC 1.17.4.2), also from microorganisms, have a different organization. The enzymes are monomers, approximately the size of the monomer of B1. Instead of the small subunit of class I enzymes, adenosylcobalamin functions as a radical generator during catalysis (14).

Recent genetic (15, 16) and biochemical (17, 18) evidence pointed to the possible existence of a fourth class, present in microorganisms during anaerobic growth. Extracts from an oxygen-sensitive mutant of *E. coli*, lacking normal class I reductase activity, catalyzed an oxygen-sensitive reduction of ribonucleotides, different from the reaction catalyzed by the known aerobic *E. coli* reductase (18). A similar activity was found in extracts from the strict anaerobic *Methanobacterium thermoautotrophicum* (17). We now describe fractionation and initial characterization of the ribonucleotide reductase system from anaerobic *E. coli*. The overall reaction requires the presence of several proteins and low molecular weight components. *S*-Adenosylmethionine (SAM) was identified as a cofactor. The highly purified ribonucleotide reductase proper is immunologically related to protein B1 of the aerobic reductase.

MATERIALS AND METHODS

Materials. SAM and *S*-adenosylhomocysteine were from Boehringer Mannheim and lysine-specific protease (*Achromobacter lyticus*) was from Wako Chemicals (Neuss, F.R.G.). Polyclonal rabbit antibodies against protein B1 were a gift from Britt-Marie Sjöberg (Stockholm University), monoclonal antibodies antiB1-c and antiB1-g were available in this laboratory (19). dATP-Sepharose was prepared by coupling aminophenyl-γ-dATP (20), kindly prepared for us by J. Ludwig (Max Planck Institut für Medizin, Göttingen, F.R.G.), to CNBr-activated Sepharose (21). Sources of other materials were given earlier (18).

Fractionation of Ribonucleotide Reductase Activity. Extracts from the oxygen-sensitive derivative OxyS-11 of *E. coli* K-12 strain AB1157 were prepared as described (18) in a Mark 3 anaerobic workstation (Don Whitley, Scientific Limited, Shipley, England) kept at +4°C. After centrifugation, the extract was passed through a column of Sephadex G25,

Abbreviation: SAM, *S*-adenosylmethionine.

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equilibrated with buffer A (20 mM Tris·HCl, pH 7.5/2 mM dithiothreitol). The resulting fraction SE (see Fig. 1) was frozen and kept under liquid nitrogen. After thawing, it was slowly introduced into a column of dATP-Sepharose (40–100 mg of protein per ml). The flow-through was collected as fraction dA1. The column was then washed with 0.5 M NaCl in buffer A until the protein concentration of the eluate was below 0.1 mg/ml, followed by 2–3 column vol of buffer A. Fraction dA3 was then eluted with 2 mM ATP in buffer A.

Fractions LM 55 and LM 100, respectively (cf. Fig. 1), were obtained by heating a small volume of the bacterial extract at 55°C for 5 min or at 100°C for 1 min.

Assays for CTP reduction were made as described earlier, with CTP replacing CDP as the substrate (18). GTP reduction was assayed as described by Barlow (22).

Peptide Maps of α, β, and Protein B1. Two proteins, α and β, from fraction dA3 were separated by preparative electrophoresis on denaturing gels. The corresponding bands were cut out and electroeluted, and the proteins, after reduction with dithiothreitol, were carboxymethylated with excess [2-^{14}C]iodoacetate in 6 M guanidinium chloride. Protein B1 was carboxymethylated in the same way. After removal of low molecular weight material by passage through Sephadex G25, equilibrated with 30% acetic acid, the proteins were lyophilized, redissolved in a small vol of 1 M urea, and digested with a lysine-specific protease (1 part enzyme per 25 parts protein). The digested material was then separated directly by HPLC on a C18 column as described in the legend to Fig. 4.

RESULTS

Fractionation of Reductase Activity. Crude extracts of anaerobically grown *E. coli* catalyzed the reduction of CTP to dCTP in the presence of ATP, NADPH, Mg^{2+}, dithiothreitol, and phospho*enol*pyruvate (18). If air was present during preparation of the extract or during incubation, the reaction did not occur. Purification of the activity was therefore started in an anaerobic box maintained at +4°C.

ATP stimulated and dATP inhibited CTP reduction by the extract (18), suggesting that the enzyme is an allosteric protein. We thought that affinity chromatography on dATP-Sepharose would provide a suitable purification step, as is the case with the allosteric aerobic enzyme. However, when in preparation for such a step the crude extract was passed through a Sephadex G25 column, the resulting fraction SE (Fig. 1) completely lacked activity, suggesting requirements for low molecular weight cofactors. Addition of the inactive fraction LM 55, obtained by heating the bacterial extract for 5 min at 55°C, restored the activity of SE (Fig. 1). Complementation by LM 55 thus provided an assay for the purification of the CTP reductase activity of SE.

Further purification of this activity by chromatography on dATP-Sepharose resulted in the separation of two fractions, the run-through fraction dA1 and fraction dA3, eluted from the column with ATP (Fig. 1). In the presence of LM 55, CTP reduction was absolutely dependent on dA3, whereas the requirement for dA1 was relative and was pronounced only at low concentrations of LM 55 (Fig. 1 *Lower Center*). dA3 turned out to be the ribonucleotide reductase (see below). dA1 is a composite protein fraction that was also present in LM 55. Heating fraction LM 55 to 100°C destroyed its activity and the resulting fraction LM 100 only poorly complemented the activity of either SE or the combined fractions dA1 and dA3. Clearly one of several of the low molecular weight components present in LM 55 was heat labile. This heat-labile compound was identified as SAM.

Reduction of CTP required the presence of four fractions as depicted in Fig. 2. Two contain proteins (dA1 and dA3), and two are low molecular weight compounds (SAM and LM 100). dA3 in all probability represents the anaerobic ribonu-

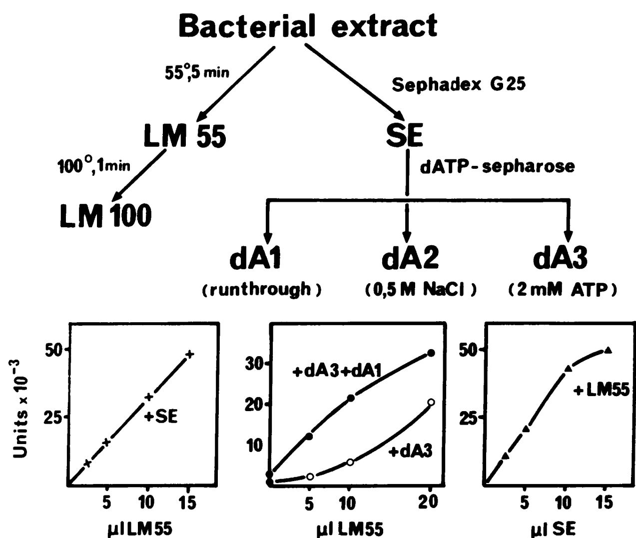

FIG. 1. (*Upper*) Scheme for the separation of the various fractions required for the anaerobic reduction of CTP. (*Lower Left*) Dependence of the reaction on the amount of fraction LM 55 in the presence of 15 μl of SE (0.18 mg of protein) is shown. (*Lower Center*) Dependence on LM 55 in the presence of 2 μg of dA3, with (●) and without (○) 250 μg of dA1 is shown. (*Lower Right*) Dependence of fraction SE on LM 55 is shown. All incubations were for 20 min at ambient temperature in a final vol of 50 μl and in the presence of 1 mM [^{3}H]CTP (40 cpm/pmol), 2 mM ATP, 20 mM phospho*enol*pyruvate, 8 mM $MgCl_2$, 1 mM NADPH, and 5–10 mM dithiothreitol. Incubations were in small tubes under a stream of argon (18). The reaction was terminated with 0.5 ml of 1 M $HClO_4$ and the amount of dCTP formed was determined. One unit corresponds to 1 nmol of dCTP formed per min.

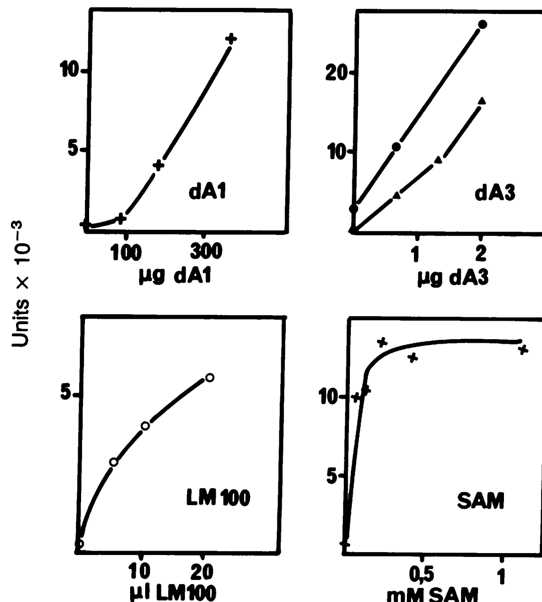

FIG. 2. CTP reduction requires two protein fractions, one low molecular weight fraction, and SAM. General conditions for incubation are given in Fig. 1. (*Upper Left*) Dependence on dA1 in the presence of 1 μg of dA3, 10 μl of LM 100, and 1 mM SAM. (*Upper Right*) Dependence on dA3 in the presence of 1 mM SAM and either 10 μl of LM 100 plus 340 μg of dA1 (●), or 20 μl of LM 100 plus 170 μg of dA1 (▲). (*Lower Left*) Dependence on LM 100 in the presence of 1 μg of dA3, 170 μg of dA1, and 1 mM SAM. (*Lower Right*) Dependence on SAM in the presence of 1 μg of dA3, 340 μg of dA1, and 5 μl of LM 100.

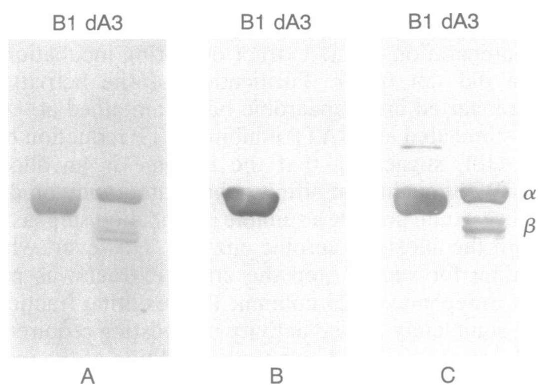

FIG. 3. SDS gel electrophoresis of protein B1 and fraction dA3. Each lane contained 3 μg of total protein. Electrophoresis was in a Bio-Rad Miniprotean II instrument for a total of 90 min. The dye marker ran off the gel after 30 min. (*A*) Proteins were stained with Coomassie brilliant blue. (*B*) Proteins were stained with a monoclonal antibody against B1. (*C*) Proteins were stained with a polyclonal antibody against B1.

cleotide reductase proper, and in the presence of an excess of the other three components the reaction was proportional to the amount of dA3 present in the system. This was not the case with dA1. For this fraction, the reaction rate showed an S-shaped dependence on the amount, suggesting that dA1 provided more than one protein required for CTP reduction.

Thioredoxin (23) and glutaredoxin (23) did not substitute for dA1 (data not shown). The reaction showed an absolute requirement for LM 100 and was strongly stimulated by SAM. The extent of stimulation by SAM depended on the amount of LM 100; it decreased in the presence of excess LM 100 and became almost absolute with suboptimal amounts of LM 100 (Fig. 2). *S*-Adenosylhomocysteine did not substitute for SAM but inhibited the reaction (data not shown).

Preliminary experiments indicate that fraction LM 100 contributes at least two factors required for CTP reduction. Treatment of LM 100 with Chelex removed one factor that was recovered from the resin by elution with HCl. This suggests the requirement for a metal.

Characterization of Fraction dA3. Analyses by denaturing gel electrophoresis (Fig. 3) revealed two major protein bands, stained with Coomassie brilliant blue: one (α) with a mobility corresponding to 90 kDa, and the other (β) with a mobility corresponding to 60–70 kDa. The β band was separated into three subbands (with increasing mobility: β1, β2, and β3). On Western blots the α band and all β bands were heavily stained by polyclonal rabbit antibodies raised against protein B1 (one of the subunits of the aerobic reductase) but not by monoclonal mouse anti-B1 antibodies (Fig. 3). This suggests that both α and β are immunologically related to B1.

To further investigate these interrelationships, we compared the peptide patterns of carboxymethylated α, combined β1 to β3, and B1 obtained after digestion with a lysine-specific protease. Fig. 4 schematically outlines the

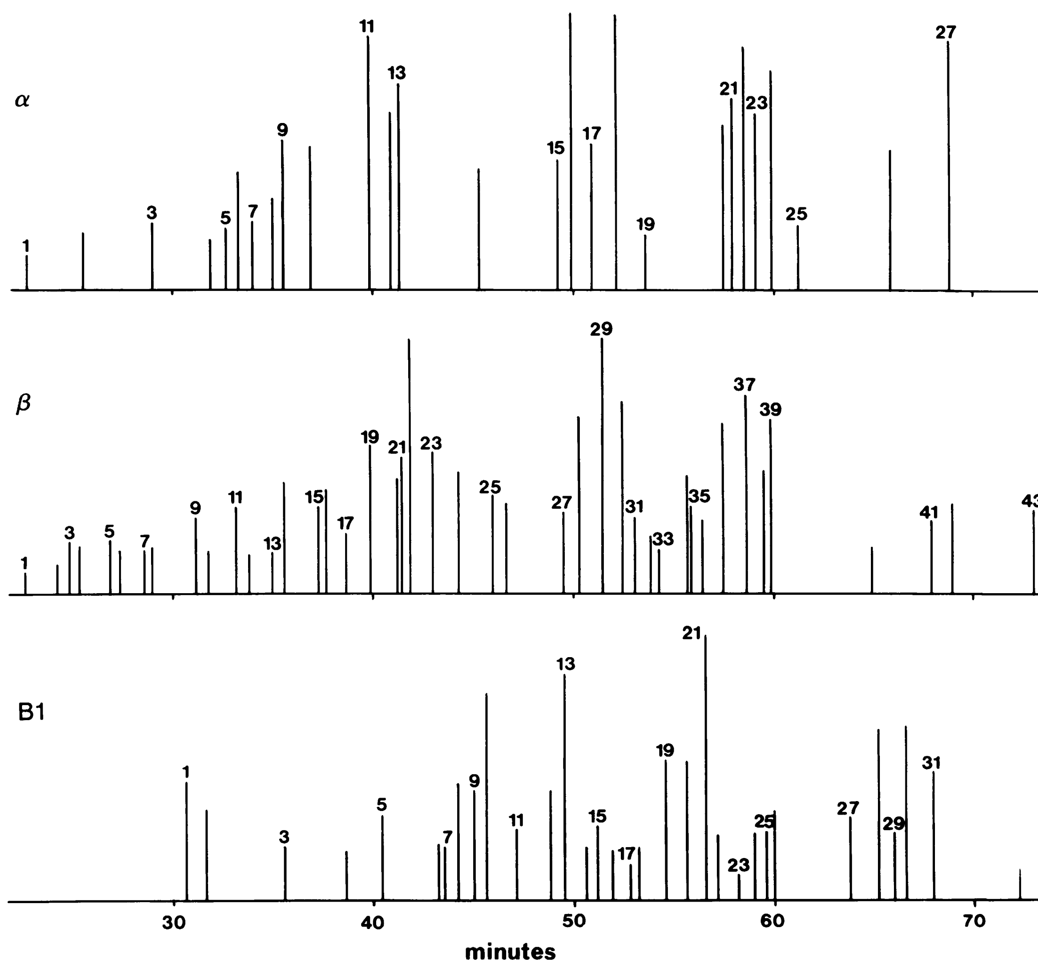

FIG. 4. Reversed-phase HPLC of peptides obtained from α, β, and B1 by digestion with a lysine-specific protease. Chromatography was on a C18 (Vydac) 218 TP 546 column with a linear gradient of 0–60% acetonitrile in 0.1% aqueous trifluoroacetic acid for 90 min at 1 ml/min. Peaks were recorded at 214 and 280 nm. The graph shows schematically the positions and heights of the well-resolved 214-nm peaks. Each run contained peptides obtained by digestion of between 29 and 31 μg of protein.

separation of the peptides by reversed-phase HPLC. Eleven pairs of peptides from α and β were eluted at identical positions and, whenever relevant, showed closely matched ratios between the absorbances at 280 and 214 nm (Table 1). In addition, relative peak heights between corresponding α/β peaks were the same (Table 1). The data suggest that many, if not all, of the 11 peptides in Table 1 are identical in α and β and form part of a common structure. Furthermore, two of the α/β pairs (α_{11}/β_{19} and α_{16}/β_{28}) were found to have at least 10-residue segments of identical amino acid sequence (data not shown). However, although β was the smallest protein, it gave rise to a considerably larger number of defined peaks than α or B1 and this complexity suggests that the β bands are not merely breakdown products of the α protein. We will return to this point in the *Discussion*. Finally, B1 clearly presents a peptide pattern that is different from those of α and β (Fig. 4).

Anaerobic Reductase Also Reduces GTP. In earlier and present work, we use the reduction of CTP to dCTP to monitor the anaerobic enzyme. Activity was observed only under strict anaerobiosis. In contrast, Barlow (22) reported that the reduction of a different nucleotide (GTP) in extracts from anaerobic *E. coli* was insensitive to oxygen. We next investigated the reduction of GTP under anaerobic and aerobic conditions and also tested the influence of phospho*enol*pyruvate on the reaction, since we had found that it stimulated CTP reduction. As shown in Table 2, GTP reduction was indeed dependent on strict anaerobiosis and was stimulated by phospho*enol*pyruvate. We do not understand the discrepancy between the earlier (22) and our present work. Possibly the earlier report monitored the transfer of guanine from ribose to deoxyribose rather than ribonucleotide reduction, a complication recently encountered in other work (24). Conditions for the substrate specificity of anaerobic ribonucleotide reduction will eventually be worked out with a pure enzyme but the present results already indicate that both pyrimidine and purine ribonucleotide reduction by the anaerobic *E. coli* reductase is oxygen sensitive.

DISCUSSION

The requirement for SAM and a loosely bound metal as cofactors distinguishes the anaerobic ribonucleotide reductase from *E. coli* and places the enzyme into a fourth class of reductases. We suspect that class IV enzymes will be found in other anaerobic organisms. A good candidate may be the enzyme from *M. thermoautotrophicum* (17). Class IV enzymes need not be restricted to prokaryotes, nor need all anaerobic reductases from prokaryotes necessarily belong to this class. Ribonucleotide reductases from clostridia apparently use adenosylcobalamin as a cofactor and thus are class III enzymes.

SAM is best known as a methyl donor but also appears during anaerobic metabolism in reactions that involve radical mechanisms. The anaerobic dissimilation of pyruvate is one such case. The active form of pyruvate formate lyase contains a protein-bound free radical whose enzymatic generation requires the participation of SAM (25, 26). A second case is the anaerobic lysine 2,3-aminomutase reaction (27–29). The enzyme catalyzing this reaction requires, in addition to SAM, iron for activity. This case is of particular interest in the present context since β-lysine 5,6-aminomutase, an enzyme that catalyzes an identical chemical reaction, uses adenosylcobalamin as cofactor (30) suggesting that SAM plus metal and adenosylcobalamin are functionally interchangeable. Class III and class IV ribonucleotide reductases may present a similar case.

The anaerobic ribonucleotide reductase system from *E. coli* has been separated into two protein and several nonprotein fractions. Of the proteins, α and β of fraction dA3 are clearly the best candidates for an anaerobic reductase. Both have components that are structurally and immunologically related. Furthermore, dA3 is strongly retained by dATP-Sepharose, a property expected from an allosteric reductase. There are at least three distinguishable subspecies of β. Their relative amounts varied greatly in different preparations of dA3. Catalytic activity was closely linked to the amount of $\beta 2$, while inactive preparations usually contained large amounts of β_3 together with α. β and α have many peptide sequences in common (Table 1), but each protein clearly also contains unique sequences (Fig. 4). From these results, we tentatively propose that β is a composite fraction. It contains in part degraded α and in part a genuine structural component of the reductase.

The second protein fraction (dA1) probably contains several active components. From the nature of the catalytic process we would predict that this fraction contains a required reducing system [such as thioredoxin and/or glutaredoxin (23) with associated proteins]. However, neither thioredoxin nor glutaredoxin substituted for dA1 and this fraction should therefore contain an additional protein required for CTP reduction, possibly involved in the activation of the anaerobic reductase, similar to the situation with pyruvate formate lyase (25, 26). Much further work is required to define the various proteins of the anaerobic reductase system and to delineate their interactions. Already at this stage, however, it is reasonable to assume that the system, like the others, will work via a radical mechanism and that SAM, together with a metal, plays a crucial role in the process.

Table 1. Comparison of α and β peaks with identical HPLC positions (see Fig. 3)

Peak no.		Relative peak* height (214 nm)		280 nm/214 nm†	
α	β	α	β	α	β
6	11	0.45	0.50	0.00	0.00
7	12	0.26	0.26	0.00	0.00
9	14	0.58	0.75	0.040	0.034
11	19	1.00	1.00	0.11	0.11
12	20	0.68	0.78	0.050	0.055
13	21	0.72	0.92	0.035	0.038
16	28	1.07	1.26	0.034	0.028
18	30	1.07	1.43	0.048	0.033
19	32	0.29	0.33	0.26	0.21
23	38	0.62	0.77	0.00	0.00
24	39	0.86	1.14	0.086	0.080

*Size of a given peak in relation to size for α peak 11 and β peak 19, respectively.
†Ratio of peak height at A_{280}/peak height at A_{214}.

Table 2. Reduction of GTP by an anaerobic *E. coli* extract

	Units of activity × 10^{-3}	
	Anaerobic	Aerobic
Phospho*enol*pyruvate present	70	2
Phospho*enol*pyruvate absent	17	2

Incubations (22) were made at ambient temperature with 1.2 mg of protein in a final vol of 0.1 ml with 1 mM [^{3}H]GTP (3400 cpm/nmol), 0.5 mM dTTP, 8 mM $MgCl_2$, 5 mM dithiothreitol, and, where indicated, 20 mM phospho*enol*pyruvate, either under argon or in air. After 20 min, the reactions were stopped by addition of 0.3 ml of 1 M perchloric acid. After centrifugation, the supernatant solutions were neutralized with 4 M KOH and, after removal of $KClO_4$, treated with alkaline phosphatase for 90 min at 37°C. Portions of the boiled solutions were then subjected to reversed-phase HPLC to separate radioactive deoxyguanosine from guanosine (22). One unit of activity is 1 nmol of deoxyguanosine formed per min. When the mixtures were not treated with phosphatase, the activity was <1 unit in all cases.

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