Proceedings of the National Academy of Sciences
Vol. 65, No. 2, pp. 430–437, February 1970

Peptide Chain Termination, VI. Purification and Site of Action of *S*

J. Goldstein, G. Milman,* E. Scolnick, and T. Caskey

LABORATORY OF BIOCHEMICAL GENETICS, NATIONAL HEART INSTITUTE, NATIONAL INSTITUTES OF HEALTH, BETHESDA, MD.

Communicated by Marshall Nirenberg, November 7, 1969

Abstract. Peptide chain termination is a result of at least two events: terminator codon recognition and hydrolysis of peptidyl tRNA. A protein factor *S*, isolated from the supernatant of *Escherichia coli* B, stimulates fMet release. Factor *S* lowers the K_m for terminator trinucleotides without altering the V_{max} of release and therefore acts at terminator codon recognition. The *S* protein differs from initiation factors, elongation factor *G*, several forms of elongation factor *T* and release factors. The importance of the 2% *Tu* content in purified *S* is unresolved.

Introduction. Release of formyl-methionine from fMet-tRNA·AUG·ribosome intermediates is analogous to peptide release during chain termination.[1] One of two codon specific protein *R* factors and a corresponding trinucleotide codon (R_1, UAA, or UAG; R_2, UAA, or UGA) is required for release.[2] An additional protein factor, *S*, which stimulates release by either R_1 or R_2 was recently identified.[3] We now report the purification, characterization, and site of action of *S* in peptide chain termination.

Materials and Methods. Determination of *S* activity: Formyl-methionine release[1] dependent on *S* is determined in reactions containing *R* factor and terminator codon. Each reaction is incubated at 30°C and contains in a final volume of 0.050 ml: 4–6 pmoles of f[^{3}H]Met-tRNA^f·AUG·ribosome complex[1]; 0.5 nmole of terminator trinucleotide unless otherwise stated; 56–264 nunits (0.2–2.0 μg protein) R_1 (fraction VI) or R_2 (fraction VII)[3]; 0.05 *M* Tris-acetate, pH 7.2; 0.03 *M* magnesium acetate; 0.05 *M* potassium chloride; and *S*. One unit *R* activity releases 1 μmole fMet per minute under previously described conditions.[3] Except for Figure 1, all values are measurements of rate.

***S* purification:** All steps are performed at 0–4°C. A 55–80% ammonium sulfate fraction is prepared from the supernatant fraction of an *E. coli* B (2 lb, Grain Processing Co.) extract as previously described.[3] The precipitated protein, containing *S* (fraction 1, 6100 mg protein), is suspended in 44 ml of buffer *A* (0.01 *M* Tris-chloride, pH 7.5; 0.01 *M* ammonium acetate; 0.01 *M* magnesium acetate; and 0.002 DTT), and applied to an 85 × 5 cm column packed with Sephadex G-100 (bead form) equilibrated in buffer *A*. Each 18 ml fraction is eluted with buffer *A* at a flow rate of 100 ml per hour. *S* is identified in tubes 32–42 (fraction II). Fraction II (3400 mg protein) is adjusted to 411 ml in a final buffer concentration of 0.02 *M* Tris-chloride, pH 8; 0.15 *M* KCl; and 0.002 *M* DTT (buffer *B*). Following application of fraction II to a DEAE-Sephadex A-50 column (94 × 2.5 cm) equilibrated with buffer *B*, 13 ml fractions are eluted by 1000 ml of buffer *B* (36 ml/hr) followed by 1500 ml linear gradient of potassium chloride (0.15 *M* with 0.02 *M* Tris-chloride, pH 8, to 0.45 *M* with 0.02 *M* Tris-chloride, pH 7.0). The tubes containing

S activity are pooled (fraction III). Fraction III (220 mg protein in 50 ml) is dialyzed against buffer *C* (0.01 *M* imidazole, pH 6; 0.05 *M* KCl; and 0.002 *M* DTT) and applied to a 22 × 2.0 cm column packed with CM-Sephadex equilibrated in buffer *C*. Following application of fraction III, fractions (7.0 ml) are eluted with 125 ml of buffer *C* (30 ml/hr) followed by 150 ml linear gradient of potassium chloride (0.05–1.2 *M*). The tubes containing *S* activity are pooled (fraction IV). Fraction IV (4.2 mg protein in 3.0 ml) is dialyzed against buffer *D* (0.01 *M* potassium phosphate, pH 7.1; and 0.002 *M* DTT) and applied to a 8 × 0.9-cm column packed with hydroxylapatite equilibrated in buffer *D*. Following application of fraction IV, the 1.5 ml fractions are eluted (60 ml/hr) with 20 ml, 0.01 *M*; 20 ml, 0.045 *M*; and 20 ml, 0.10 *M* potassium phosphate and 0.002 *M* DTT. Tubes 8–12, 15–19, and 32–37 are separately pooled, dialyzed against buffer *E* (0.02 *M* Tris-chloride, pH 8; and 0.002 *M* DTT), and concentrated by pressure filtration (Amicon Co., Lexington, Mass.). Only tubes 32–37 contain *S* activity (fraction V). All pooled fractions of *S* are dialyzed against buffer *E*, concentrated by pressure filtration, and stored at −170°C. Details of *S* purification are given in Table 1.

Determination of elongation factor activities: Two methods of determining *T*(*Tu* + *Ts*), *Tu*, and *Ts* activities are used: (*1*) retention of [^{3}H]GTP by *T*(*Tu* + *Ts*) and *Tu* on millipore filters and the stimulation of the rate of retention by *Ts*;[4] (*2*) polymerization of phenylalanine under conditions requiring the addition of *T*(*Tu* + *Ts*), *Tu*, or *Ts*.[5] Factor *G* (translocase) activity is determined by phenylalanine polymerization dependent upon added *G* factor.[5] Conditions for phenylalanine polymerization correspond to those previously described.[3]

Elongation factors *Tu* and *Ts* purification: The major peak of *T*(*Tu* + *Ts*) obtained from the DEAE-Sephadex fractionation of *S* (tubes 130–140, Fig. 4; fraction VI), is further purified by a modification of the procedure of Miller and Weissbach which employs a DEAE-Sephadex column equilibrated in 5 × 10^{-5} *M* GTP, followed by Sephadex G-100 chromatography in the absence of GTP.[6] This fraction (fraction VII) is apparently homogenous by acrylamide gel analysis, and is stimulated 16-fold by *Ts* in phenylalanine polymerization (0.06 to 0.99 pmole polyphenylalanine), and therefore corresponds to *Tu*. Factor *Ts* is obtained from the 0.15 *M* potassium chloride wash of the DEAE-Sephadex column used in the preparation of *S* (tubes 20–125, Fig. 4).

Protein determinations are by a modification of the method of Lowry.[7] Isotopes used in these studies are L-[^{3}H methyl]-methionine (5.1 c/mm, Schwarz BioResearch Corp.); L-[^{3}H]phenylalanine (5.3 c/mm, New England Nuclear Corp.), and [^{3}H]GTP (1.16 c/mm, Schwarz BioResearch Corp.).

Results. Stimulation of formyl-methionine release: The effect of *S* on peptide chain termination is shown in Figure 1. Preparations of *S* are devoid of

TABLE 1. *Purification of S.*

	Fraction	Protein (mg)		*S* Activity		*Tu* Activity	
		Applied	Recovered	Specific activity	Recovery	Specific activity	Recovery
II	Sephadex G-100	6100	3400	1.7	1.00	140	1.00
III	DEAE-sephadex	3400	480	8.7	0.88	185	0.23
IV	CM-sephadex	220	18.3	45.0	0.39	110	0.012
V	Hydroxylapatite	4.2	0.8	67.0	0.11	47	0.001

The details of the purification of *S* are given in **Materials and Methods.** The activity of *S* was determined by fMet release at 30°C for 10 min in 0.050 ml reactions containing: 0.5 nmole UAG; 264 nunits R_1; *S* fractions as indicated; and additional components described in **Materials and Methods.** The activity of *Tu* was determined by phenylalanine polymerization. Each reaction was incubated at 30°C for 10 min and contained in 0.100 ml: saturating levels of partially purified *Ts* (6.6 μg) and *G* (1.1 μg); *S* fractions as indicated; and additional components described in **Materials and Methods.** Specific activity is the number of μunits of *S* activity or *Tu* activity per milligram *S* protein. One μunit of activity corresponds to the amount (mg) of *S* which releases one pmole of fMet per minute or *Tu* which polymerizes one pmole of phenylalanine per minute. Recovery represents a cumulative value of the ratio of input to recovered activity at each purification step.

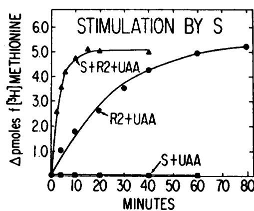


FIG. 1.—S protein stimulation of fMet release. Each reaction was incubated at 30°C for the indicated time and contained in 0.050 ml: 2.5 nmole UAA; 260 nunits R_2 as indicated; 15.2 μg S (fraction III) as indicated; and additional components described in **Materials and Methods.** fMet extracted in the absence of UAA (0.15 pmole) is subtracted from all values.

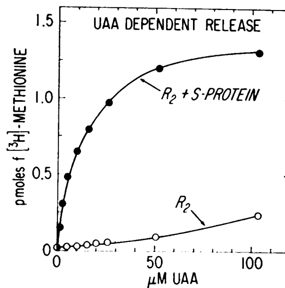
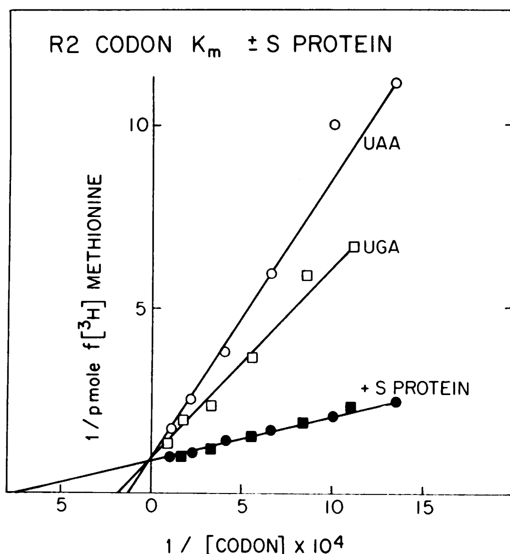


FIG. 2.—Rate of fMet release dependent on trinucleotide. Each reaction was incubated at 30°C for 10 min and contained in 0.050 ml: 107 nunits R_2; 16.0 μg S (fraction IV) as indicated; UAA as indicated, and additional components described in **Methods and Materials.** fMet extracted at zero time (0.15 pmole) is subtracted from each value.

R activity. S stimulates the rate of fMet release in the presence of R and terminator trinucleotide (only R_2 and UAA shown). The addition of GTP, GDP, or GOPOPCP (10^{-4} M) to reactions containing $S + R_2 +$ UAA inhibits only the S stimulation of release and has no effect on release of fMet dependent on $R_2 +$ UAA.[3]

At limiting concentrations of UAA, the rate of fMet release is determined in the presence and absence of S (Fig. 2). At low levels of trinucleotide, the addition of S significantly stimulates the rate of release. The trinucleotide concentration becomes saturating between 50 and 100 μM in reactions containing S. The K_m for trinucleotide codons and reaction V_{max} are determined by examining similar data by the method of Lineweaver and Burk (Fig. 3).[8] S has no effect on the V_{max} for fMet release. In the absence of S, the K_m for UAA and UGA is 8.3×10^{-5} M and 5.6×10^{-5} M, respectively. The K_m for both codons is lowered to 1.3×10^{-5} M with $S + R$. Additional experiments with R_1 indicate S lowers the K_m for UAA and UAG by six- to sevenfold. These studies suggest S affects terminator codon recognition, not hydrolysis of peptidyl tRNA.

Since S lowers the K_m for terminator codons, *in vitro* conditions which require S for release are established by using a low level of trinucleotide, 10 μM UAA (see Fig. 2). Although the effects of S are observed from 0° to 40°C in the presence of NH_4^+ or K^+, the S dependency is maximal when K^+ is used as the cation and reactions are incubated at 30°C. Using these conditions, the rate of fMet formation as a function of the concentration of S protein is shown in Figure 4. Formyl-methionine release is low (0.15 pmole/12 min) without S at 10 μM UAA and saturating R_1. The addition of S to these reactions increases the rate of release, proportional to S protein concentration, up to 1.8 pmole/12 minutes. These conditions provide a highly sensitive method for detection and quantitation of S activity.



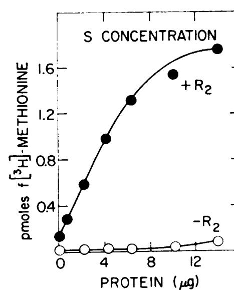


FIG. 4.—S-dependent release. Each reaction was incubated at 30°C for 12 min and contained in 0.050 ml: 0.5 nmole UAA; 56 nunits R_2 as indicated; S (fraction IV) as indicated; and additional components described in **Materials and Methods.** fMet extracted (0.28 pmole) at zero time is subtracted from all values.

FIG. 3.—K_m determination for UAA and UGA. Each release assay was incubated at 24°C for 10 min and contained in 0.050 ml: 107 nunits R_2; 6.3 μg S (fraction IV), as indicated by closed symbols(●,■); UAA (○) or UGA (□) as indicated, and additional components described in **Materials and Methods.** fMet extracted in the absence of trinucleotide codon (0.14 pmole) is subtracted from all values.

Purification of S: Both the 0–55 and 55–80 per cent ammonium sulfate fractions of *E. coli* supernatant extracts contain S. Since the 55–80 per cent fraction is devoid of R activity[2] and the chromatographic behavior and stimulatory characteristics of S isolated from the two ammonium sulfate fractions do not appear to differ, we have purified S from the 55–80 fraction (fraction 1). Following ammonium sulfate precipitation, fraction I is chromatographed on Sephadex G-100 (fraction II). Although S activity is detected in fraction I,[3] fraction II is the earliest preparation which gives reliable specific activity determinations. S is further purified by DEAE-Sephadex chromatography (Fig. 5). A single peak of S activity is identified (tubes 144–154, fraction III) with R_1 and UAA or UAG, or R_2 and UAA or UGA. The elongation factor activities of the column fractions are also determined. S separates from Ts (tubes 20–125, not illustrated); G (tubes 157–170); and the major $T(Tu + Ts)$ peak (tubes 130–140). Fractions containing S (tubes 144–154) also contain T activity (determined by both GTP binding and Tu-dependent phenylalanine polymerization). Although S and the minor peak of T appear to coincide in Figure 5, on other occasions S eluted between the two T peaks. S is further purified by CM-Sephadex column chromatography (Fig. 6). The S activity elutes with 0.7 M KCl (fraction IV). Two fractions of T activity are detected (determined both by GTP binding and polymerization); tubes 3–8 with no detectable S activity and tubes 27–31 coinciding with S activity. S is further purified by hydroxylapatite column chromatography (Table 1, fraction V). The T activity of this fraction is stimulated 12-fold by Ts in phenylalanine polymerization (0.05 to 0.60 pmole polyphenylalanine)

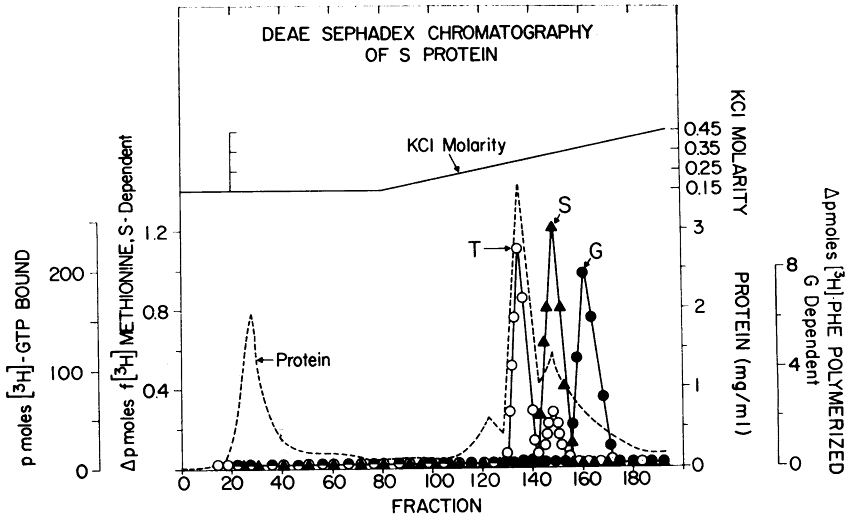


FIG. 5.—DEAE-Sephadex chromatography of S. The details of chromatography are given in **Materials and Methods.** S (fraction II, 1170 mg protein) was eluted from DEAE-Sephadex by a linear potassium chloride gradient (1500 ml, 0.15–0.45 M potassium chloride). S activity (▲) was determined by fMet release at 30°C for 25 min due to 0.005 ml addition of indicated column fractions to reactions containing in 0.050 ml: 0.5 nmole UAA; 107 nunits R_2; and additional components described in **Materials and Methods.** fMet extracted without additions of column fractions (0.41 pmole) is subtracted from all values. Transfer activity T (○) was determined by retention of [^{3}H]GTP to Millipore filters at 0°C for 5 min by 0.010-ml portions of the indicated fractions. Translocase activity G (●) was determined by phenylalanine polymerization at 30°C for 10 min by 0.010 ml addition of the indicated fractions to reactions which require G for polymerization. Phenylalanine polymerized without additions (0.23 pmole) is subtracted from each value.

and therefore resembles Tu. The S activity of this fraction is unaffected by the addition of Ts.

A summary of the purification of S and the Tu content of each fraction is given in Table 1. S is purified 40-fold with 11 per cent cumulative recovery of activity. (If the specific activity of fraction I rather than fraction II is compared to fraction V, S is purified more than 220-fold.) Tu specific activity in S decreases threefold with 0.1 per cent recovery of total activity. Although $T(Tu + Ts)$ does not correspond to S (Figs. 5 and 6), we have purified Tu in order to investigate the possibility that S corresponds to Tu. The S and Tu activities of S (fraction V) and homogeneous Tu (fraction VII) are compared in Figure 7. Both this fraction of Tu and a second preparation of highly purified Tu isolated by a different method[9] have no detectable S activity and do not stimulate or inhibit S dependent release. Fraction V of S contains 2 per cent Tu protein as determined by specific activity comparisons with pure Tu. Levels of S which are saturating for formyl-methionine release severely limit Tu-dependent protein synthesis. These studies suggest that Tu does not correspond to S.

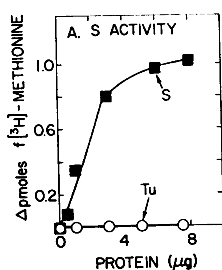
Discussion. The S protein is purified from the supernatant fraction of *E. coli* extracts and separates from initiation factors F_1, F_2, and F_3[10] (unpublished data), elongation factors Ts, $T(Tu + Ts)$, and G,[5] and release factors R_1 and R_2.[2]

FIG. 6.—CM-Sephadex chromatography of S. The details of chromatography are given in **Materials and Methods.** S (fraction III, 69 mg protein) was eluted from CM-Sephadex by a linear potassium chloride gradient (150 ml, 0.05–1.20 M KCl). S activity (▲) was determined by fMet release at 30°C for 10 min due to 0.010 ml addition of dialyzed (buffer E) column fractions to reactions containing in 0.050 ml: 0.5 nmole UAA; 107 nunits R_2; and additional components described in **Materials and Methods.** fMet extracted without additions of column fractions (0.35 pmole) is subtracted from each value. Transfer activity T (○) was determined by retention of [^{3}H]GTP to Millipore filters at 37°C for 5 min by assay of 0.050 ml portions of indicated column fractions.

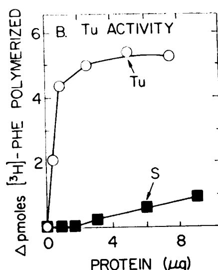
Although S protein preparations contain Tu activity (see *Results*), the Tu content of S diminishes in total and specific activities throughout purification. More extensive purification of S is needed to resolve whether the 2 per cent Tu content in S occurs as a contaminant or is essential for S activity. However, two highly purified preparations of Tu have no effect on fMet release by R in the presence or absence of S. The available data indicate that S does not correspond to the previously characterized protein factors involved in peptide chain initiation, elongation, and termination,[11, 12] and thus suggest S is an additional factor involved in protein synthesis.

Peptide chain termination is a result of both terminator codon recognition and hydrolysis of peptidyl tRNA and recently was shown to proceed through the formation of a release factor·terminator codon·ribosome intermediate[13] which occurs prior to peptide release. Since S-dependent fMet release is a reflection of both codon recognition and hydrolysis of peptidyl tRNA, S could act at either or both events. The release of fMet with R and terminator codon is consistent with the Michaelis-Menten theory of enzyme kinetics, where the equilibrium constant for the formation of the terminator codon recognition intermediate (R·terminator codon·70S ribosome·AUG·fMet-tRNA^F) is reflected in the trinucleotide K_m determination and the rate of hydrolysis of fMet from this intermediate is reflected in the V_{max} measurement.[14]

Since S has no effect upon V_{max} but does lower the K_m for trinucleotide codons,



(*A*) *S* activity was determined as described in **Materials and Methods.** Each reaction was incubated at 30°C for 15 min and contained in 0.050 ml: 0.5 nmole UAA; 56 nunits R_2; and indicated amounts of either *S* (fraction V) or *Tu* (fraction VII). fMet extracted in the absence of added *S* or *Tu* (0.41 pmole) is subtracted from each value.



(*B*) *Tu* activity was determined by phenylalanine polymerization at 30°C for 15 min in 0.100 ml reactions containing: saturating levels of partially purified *Ts* (6.6 μg) and *G* (1.1 μg); indicated amounts of either *S* (fraction V) or *Tu* (fraction VII); and additional components described in **Materials and Methods.** Phenylalanine polymerized without *S* or *Tu* (0.70 pmole) is subtracted from each value.

FIG. 7.—Determination of *S* and *Tu* activities.

its site of action is at codon recognition. We suggest that *S* stimulates release by facilitating the formation of the *R*·terminator codon·ribosome intermediates. This proposed action of *S* for chain termination may be analogous to the formation of fMet-tRNA·AUG·ribosome intermediates by initiation factors[15] and aa-tRNA·codon·ribosome intermediates by elongation factor *T*.[16] While it seems clear that *S* affects events during terminator codon recognition and not hydrolysis of peptidyl tRNA, the precise sequence of events and the mechanism of the GTP, GDP, or GOPOPCP inhibition requires further study.

The authors take great pleasure in acknowledging the aid of Dr. R. Tompkins and Dr. M. Nirenberg, the excellent technical assistance of Mrs. Theresa Caryk, and the invaluable help of Mrs. Exa Murray in the preparation of this manuscript.

The following abbreviations were used: CM-Sephadex, carboxymethyl-Sephadex; DEAE-Sephadex, *O*-(diethylaminoethyl-Sephadex); DTT, dithiothreitol; GOPOPCP, 5′-guanylylmethylenediphosphonate.

* Postdoctoral Fellow of the Helen Hay Whitney Foundation.

1 Caskey, C. T., R. Tompkins, E. Scolnick, T. Caryk, and M. Nirenberg, *Science*, **162**, 135 (1968).

2 Scolnick, E., R. Tompkins, T. Caskey, and M. Nirenberg, these PROCEEDINGS, **61**, 768 (1968).

3 Milman, G., J. Goldstein, E. Scolnick, and T. Caskey, these PROCEEDINGS, **63**, 183 (1969).

4 Ertel, R., N. Brot, B. Redfield, J. E. Allende, and H. Weissbach, these PROCEEDINGS, **59**, 862 (1968).

5 Lucas-Lenard, J., and F. Lipmann, these PROCEEDINGS, **55**, 1562 (1966).

6 Miller, D., and H. Weissbach, manuscript in preparation.

7 Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).

[8] Lineweaver, H., and D. Burk, *J. Amer. Chem. Soc.*, **56**, 658 (1934).
[9] Lucas-Lenard, J., P. Tao, and A.-L. Haenni, *Cold Spring Harbor Symposia on Quantitative Biology*, vol. 34, in press.
[10] Iwasaki, K., S. Sabol, A. J. Wahba, and S. Ochoa, *Arch. Biochem. Biophys.*, **125**, 542 (1968).
[11] Lipmann, F., *Science*, **164**, 1024 (1969).
[12] Lengyel, P., and D. Söll, *Bact. Revs.*, **33**, 264 (1969).
[13] Scolnick, E. M., and C. T. Caskey, these PROCEEDINGS, **64**, 1235 (1969).
[14] Dixon, M., and E. C. Webb, in *Enzymes* (New York: Academic Press, 1964), p. 63.
[15] Salas, M., M. B. Hille, J. A. Last, A. J. Wahba, and S. Ochoa, these PROCEEDINGS, **57**, 387 (1967).
[16] Ravel, J. M., these PROCEEDINGS, **57**, 1811 (1967).