# Purification of Bovine Glia Maturation Factor and Characterization with Monoclonal Antibody†

Ramon Lim,* Joyce F. Miller, Danny J. Hicklin, and Andrew A. Andresen

*Veterans Administration Medical Center and Department of Neurology, Division of Neurochemistry and Neurobiology, University of Iowa, Iowa City, Iowa 52242*

*Received May 31, 1985*

ABSTRACT: Glia maturation factor (GMF) is purified 100 000-fold to apparent homogeneity from bovine brains by a procedure consisting of ammonium sulfate precipitation, column chromatography with diethylaminoethyl-Sephacel, Sephadex G-75, and hydroxylapatite, and a final step using $C_4$ reverse-phase high-performance liquid chromatography. The product shows a single protein band in sodium dodecyl sulfate–polyacrylamide gel. It has a molecular weight of 14 000 and an isoelectric point of pH 5.2. Purified GMF stimulates cultured astroblasts to proliferate and to grow out cell processes with half-maximal activity at 8 ng/mL. A monoclonal antibody raised against partially purified GMF adsorbs the activity of pure GMF and immunologically binds the putative GMF protein band.

Glia maturation factor (GMF),[1] first detected in our laboratory (Lim et al., 1972, 1973; Lim & Mitsunobu, 1974), is an acidic protein endogenous to the adult brain of many species. GMF has been implicated in a number of cellular control mechanisms in the nervous system. It promotes the proliferation and phenotypic expression of astrocytes (Lim, 1980) and Schwann cells (Bosch et al., 1984) in culture and reverses some of the neoplastic growth properties of glial tumor cells (Lim et al., 1981). GMF also stimulates the production of other growth factors or hormones from astrocytes such as interleukin 1 (Fontana et al., 1983) and the prostaglandins (Miller & Lim, 1984). When used in vivo, GMF enhances the morphological recovery of brain tissue following injury to newborn rats (Lim & Miller, 1985). For the most recent review on GMF up to Dec 1983, see article by Lim (1985). Despite attempts to purify this biologically active substance, the chemical nature of GMF remained obscure. We last reported a 10 000-fold partial purification (Lim & Miller, 1984). We now present data on the complete purification of GMF using HPLC as the final step. A monoclonal antibody is obtained that abolishes GMF activity and binds the purified GMF protein.

## Materials and Methods

*Production of Partially Purified GMF.* A 10 000-fold partially purified GMF sample was used as the starting material for HPLC fractionation. This was prepared from bovine brains by a procedure previously described (Lim & Miller, 1984), which comprises the following steps: ammonium sulfate precipitation, DEAE-Sephacel chromatography, Sephadex G-75 chromatography, and hydroxylapatite chromatography (Table I).

*HPLC Procedure.* The HPLC column consisted of a 4.6 mm × 5 cm Vydac $C_4$ reverse-phase column (The Separations Group, Hesperia, CA) having a particle size of 5 μm and a pore size of 300 Å. Lyophilized GMF samples were dissolved in the organic solvent (acetonitrile with TFA) immediately before application to the column. The eluted fractions were collected in polypropylene tubes and lyophilized to remove the solvent. Before lyophilization, fractions were stored at −70 °C for up to 2 days. Fractions intended for cell testing were collected in tubes containing BSA to a final concentration of 0.1 mg/mL. These were likewise frozen and lyophilized before use. All other conditions were as described under Results.

*Bioassay of GMF.* GMF was assayed on confluent cultures of astroblasts derived from 17-day-old fetal rat brains. Testing was conducted in F10 medium containing 5% fetal calf serum. GMF activity was based on mitogenicity (increase in [$^3$H]-thymidine incorporation into DNA) and on morphological change (percentage of cells growing out processes), as described before (Lim & Miller, 1984).

*SDS–Polyacrylamide Gel Electrophoresis.* Lyophilized GMF samples were taken up in a sample buffer consisting of 0.1 M dithiothreitol, 2% SDS, 15% glycerol, 2 mM phenylmethanesulfonyl fluoride, 2 mM EDTA, 1 mM *N*-ethylmaleimide, 1 mM iodoacetic acid, 75 mM Tris-HCl (pH 6.8), and 0.001% bromophenol blue. After the solution was heated at 100 °C for 5 min, 20 μL was applied to each slot in a gel slab, 70 × 80 × 0.75 mm in dimension, made in a "Mighty Small" apparatus (Hoefer Scientific Instruments). The stacking gel, separation gel, and electrode buffer, all containing 0.1% SDS, were prepared according to Laemmli (1970). Electrophoresis was conducted at a constant current of 20 mA for about 2 h. The gels were fixed and stained with silver nitrate by using the kit purchased from Bio-Rad.

*Amino Acid Analysis.* Protein samples were hydrolyzed for 24 h in 6 N HCl containing 0.2% 2-mercaptoethanol and analyzed with a Beckman 121 MB amino acid analyzer. Cysteine content was determined as cysteic acid after per-

† This work was supported by the following to R.L.: VA Merit Review and Career Investigatorship Awards, NSF Grant BNS-8308341, NCI Grant CA-31796, Diabetes–Endocrinology Research Center Grant AM-25295, and University of Iowa Cancer Center Grant. This paper is dedicated to Prof. Maurice W. Van Allen, Chairman of Department of Neurology, on the occasion of his retirement.

* Author to whom correspondence should be addressed.

[1] Abbreviations: BSA, bovine serum albumin; ELISA, enzyme-linked immunosorbent assay; GMF, glia maturation factor; HPLC, high-performance liquid chromatography; IEF, isoelectric focusing; PAGE, polyacrylamide gel electrophoresis; PBS, 10 mM sodium phosphate and 0.15 M NaCl, pH 7.4; SDS, sodium dodecyl sulfate; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; TBS, 20 mM Tris-HCl and 0.15 M NaCl, pH 7.4; TPBS, 0.05% Tween 20 in PBS; TFA, trifluoroacetic acid; EDTA, ethylenediaminetetraacetic acid.

0006-2960/85/0424-8070$01.50/0 

formic acid oxidation. Threonine and serine contents were not corrected for destruction. No separate hydrolysis was carried out for tryptophan determination.

*Production of Monoclonal Antibodies.* Eight-week-old female Balb/c mice were immunized with four injections, 3 weeks apart, of 750 μg of partially purified GMF (through the hydroxylapatite step). The first injection was given subcutaneously with complete Freund's adjuvant; subsequent injections were given intraperitoneally with incomplete Freund's adjuvant. Each injection consisted of 200 μL total volume. Two days after the final inoculation the mice were tail-bled and the serum was screened for antibody against the partially purified GMF by means of enzyme-linked immunosorbent assay (ELISA), using the β-galactosidase reaction for visualization (Cobbold & Waldmann, 1981). Mice showing a positive response were sacrificed 2 days later, and the spleen cells were fused with NS1 myeloma cells by using the standard procedure (Kennett, 1980). The hybridoma colonies were screened (ELISA) for antibody production by using partially purified GMF and then pure GMF. Bovine serum albumin (BSA) was employed as a negative control antigen during screening. Following additional cycles of recloning and rescreening with ELISA, several hybrid lines were maintained. Results presented in this paper are derived from an $IgG_{2b}$ producing clone designated G2-09. The hybridoma-conditioned medium was concentrated 10-fold by filtration with Amicon XM-50 membrane before use.

*Immunoadsorption Using Monoclonal Antibody.* Varying volumes of the hybridoma-conditioned medium containing the monoclonal anti-GMF antibody (G2-09) were incubated with purified GMF in a total volume of 700 μL by using a polypropylene tube. (The amount of GMF used was adjusted to have a final concentration of 40 ng/mL medium during cell testing, in the absence of antibody.) The initial incubation was carried out at 4 °C for 3 h in Tris-buffered saline (TBS). Subsequently the mixture was incubated at 4 °C for 1 h with 200 μL of a 10% suspension of formalin-fixed *Staphylococcus aureus* precoated with excess goat anti-mouse-IgG antibody according to the procedure of Sweadner (1983); *S. aureus* was purchased from Bethesda Laboratories and treated by the method of Kessler (1975) before use. After the immunoprecipitate was removed by centrifugation, 100 μL of the supernatant was assayed for GMF activity on rat astroblasts. Results presented are the mean of quadruplicates with variability of less than 5%. If GMF was adsorbed with the primary antibody which was first attached to *S. aureus* via the secondary antibody, the same results were obtained. Omission of the secondary antibody led to less effective adsorption. Control experiments where the primary antibody was omitted or was replaced by nonantibody-producing conditioned medium did not result in the loss of GMF activity.

*Immunoblotting Using Monoclonal Antibody.* GMF samples taken from various stages of purification were subjected to SDS–PAGE or IEF and subsequently electrotransferred to nitrocellulose sheets by a method modified from Towbin et al. (1979) and Ogata et al. (1983). Briefly, the gel slab was soaked for 30 min in transfer buffer (195 mM glycine, 20% methanol, 0.1% SDS, and 25 mM Tris-HCl at pH 8.3) and placed in contact with a nitrocellulose sheet (Bio-Rad); the two were sandwiched between two pieces of double-layered Whatman 3MM paper, and the whole setup was in turn sandwiched between two Scotch-Brite scouring pads. All materials were soaked with transfer buffer before assemblage. Electroblotting was conducted at a constant current of 250 mA for 2 h in a Hoefer transfer chamber, with the nitrocellulose sheet facing the anode. After being blotted, the sheet was treated successively as follows: washing in TBS containing 1% Nonidet P-40 for 1 h; soaking overnight in TBS containing 3% BSA; washing 3 times for 15 min with PBS containing 0.05% Tween 20 (TPBS); incubation for 4 h with hybridoma-conditioned medium containing anti-GMF antibody G2-09; washing 3 times for 30 min with TPBS. Finally, the positive bands were revealed emzymatically by the use of a Vectastain ABC kit (Vector Laboratories), employing biotinylated goat anti-mouse-IgG as the secondary antibody, avidin–biotinylated peroxidase complex as the enzyme, and 4-chloro-1-naphthol as the substrate chromogen.

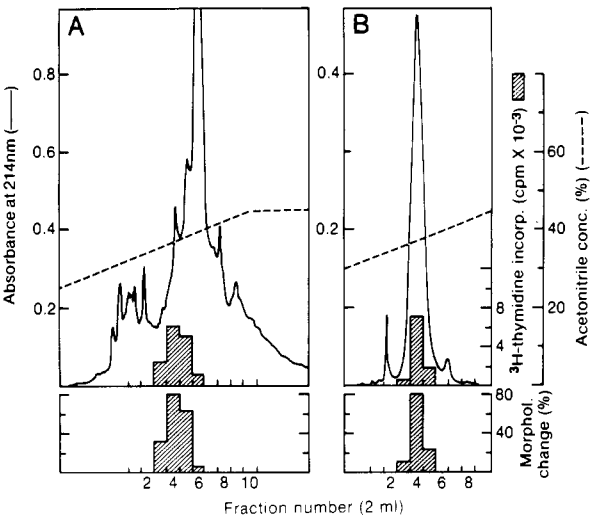


FIGURE 1: Final purification of GMF by repeated HPLC fractionation. The partially purified GMF sample was chromatographed on a reverse-phase HPLC column as described in the text. The flow rate was 1.5 mL/min. Sample 1 refers to the fraction collected 12.7 min from the start of the gradient elution. However, all fractions, including those unnumbered, were assayed for GMF activity, both mitogenic and morphological.

## RESULTS

The partially purified GMF sample obtained through the hydroxylapatite step (Lim & Miller, 1984) was subjected to two successive HPLC runs in a Vydac $C_4$ reverse-phase column (Figure 1) as follows. Forty milliliters of the hydroxylapatite-eluted material was dialyzed against 0.1 M ammonium formate and lyophilized. The dried material was taken up in 2 mL of 20% acetonitrile containing 0.1% trifluoroacetic acid (TFA) and applied to the HPLC column. The charged column was washed with 20 mL of the same solvent mixture and eluted with 40 mL of a linear gradient of acetonitrile (20–45%) containing 0.1% TFA. GMF activity was invariably detected in fractions 3–5 (Figure 1A). The corresponding fraction 4's from three parallel HPLC runs, equivalent to about 120 mL or one batch of the hydroxylapatite-purified sample (Lim & Miller, 1984), were combined, lyophilized, and redissolved in 2 mL of 20% acetonitrile containing 0.1% TFA. The pooled sample was rerun in the same HPLC column under identical conditions, and the fractions were collected as before (Figure 1B). On the second HPLC run GMF emerged as a single absorbance peak which coincided with the activity peak. Fraction 4 contained the final product. Overall purification was 100000-fold, starting from the crude brain extract (Table I). The purification fold was probably a conservative estimate, as denaturation appeared to occur during the HPLC step.

When analyzed on SDS–PAGE under reducing conditions (Figure 2), the purified GMF protein appeared as a single band, indicating the absence of disulfide-linked subunits. The

Table I: Purification of GMF from Bovine Brain[a]

| step | protein recovered (mg) | mitogenic activity: activity recovered (units) | mitogenic activity: specific activity (units/mg) | morphological activity: activity recovered (units) | morphological activity: specific activity (units/mg) |
|---|---|---|---|---|---|
| crude extract | 69 430.00 | 69 430 | 1 | 69 430 | 1 |
| $(NH_4)_2SO_4$ fraction | 6 456.00 | 66 540 | 10 | 66 540 | 10 |
| DEAE-Sephacel | 1778.00 | 65 370 | 37 | 65 370 | 37 |
| Sephadex G-75 | 30.50 | 30 031 | 985 | 29 086 | 954 |
| hydroxylapatite | 1.20 | 12 300 | 10 250 | 11 952 | 9 960 |
| HPLC | 0.03 | 3 100 | 103 333 | 2 990 | 99 667 |

[a] The data are collected on the basis of 2.8 kg (wet weight) of bovine brain as the starting material. One unit of activity is defined as that exhibited by 1 mg of protein in the crude extract. Values for purification steps through hydroxylapatite were previously published (Lim & Miller, 1984). Purified GMF protein was estimated from the intensity of silver stain on SDS–PAGE, with BSA as standard, and verified with amino acid analysis. Protein estimation in all preceding steps was based on $A_{280}^{1\%} = 10$.

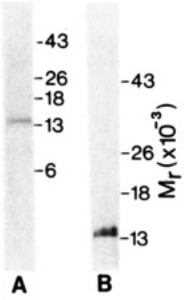


FIGURE 2: SDS–polyacrylamide gel of purified GMF: (A) 10 ng of GMF protein electrophoresed in 18% separation gel; (B) 100 ng of GMF protein electrophoresed in 12% separation gel. The stacking gel was 4% in both cases. Silver stained. Molecular weight standards are indicated on the right.

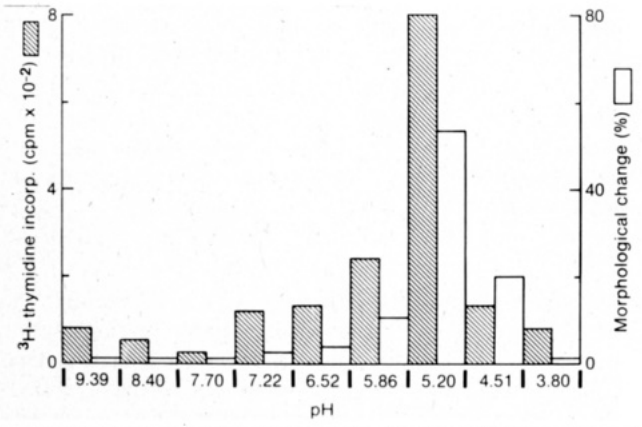


FIGURE 3: Isoelectric point determination. Purified GMF was focused on an LKB polyacrylamide plate having a pH range of 3.5–9.5. The plate was prefocused for 1 h and focused for 1.5 h, both at 15 W. Electrode solutions were 1 M $H_3PO_4$ at the anode and 1 M NaOH at the cathode. After isoelectric focusing the plate was cut into 1-cm segments and each segment soaked overnight at 4 °C with 3 mL of F10 medium containing 5% serum and 0.01% BSA. The eluate was filter-sterilized and 50 μL was taken for the assay of GMF activity. The pH of each segment was measured on water extracts of blank lanes.

molecular weight was about 14 000. The isoelectric point as determined with isoelectric focusing was pH 5.2 (Figure 3). The amino acid composition is depicted in Table II. The dose–response curves (Figure 4) showed that both the mitogenic and morphological activities reached a plateau at about 40 ng of GMF/mL of culture medium, with a half-maximal point at 8 ng/mL.

As with the cruder preparations, pure GMF stimulated process outgrowth from astroblasts and altered the histotypic pattern from a flat, confluent morphology to an interconnecting cellular network (Figure 5). This response was completely abolished when the GMF sample was first adsorbed with the anti-GMF monoclonal antibody. The progressive decrease in

Table II: Amino Acid Composition of GMF (Residues per Molecule)

| amino acid | observed | integer |
|---|---|---|
| aspartic acid | 10.2 | 10 |
| threonine | 3.88 | 4 |
| serine | 7.56 | 8 |
| glutamic acid | 21.7 | 22 |
| proline | 4.91 | 5 |
| glycine | 11.3 | 11 |
| alanine | 18.93 | 19 |
| half-cystine[a] | 1.90 | 2 |
| valine | 5.80 | 6 |
| methionine | 3.3 | 3 |
| isoleucine | 5.58 | 6 |
| leucine | 12.88 | 13 |
| tyrosine | 2.92 | 3 |
| phenylalanine | 1.71 | 2 |
| lysine | 14.3 | 14 |
| histidine | 1.02 | 1 |
| tryptophan | *b* | |
| arginine | 4.76 | 5 |
| total no. of residues | | 134 |
| calcd molecular weight | | 14 057 |

[a] Determined after performic acid oxidation. [b] Trace.

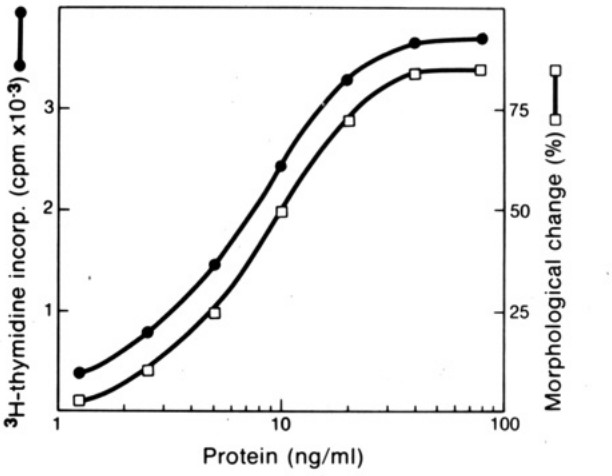


FIGURE 4: Dose–response curves of purified GMF on density-arrested rat astroblasts, showing both mitogenic and morphological activities. The cells were tested in F10 medium containing 5% fetal calf serum (Lim & Miller, 1984). Each point is a mean of four determinations with SD of 5% or less.

mitogenic and morphological activities with increasing amounts of the antibody is shown in Figure 6.

In order to verify that the monoclonal antibody was indeed directed against the putative GMF protein band, immunoblotting was conducted on pure and partially purified GMF samples. As shown in Figure 7, the antibody not only reacted with pure GMF but also selectively bound the GMF protein in the cruder preparations.

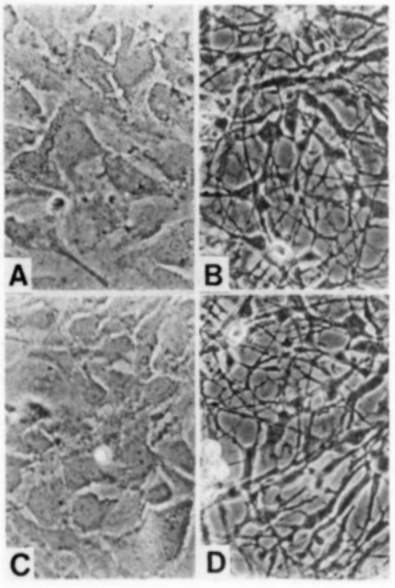


FIGURE 5: Morphological effect of purified GMF. Confluent cultures of rat astroblasts were stimulated by pure GMF in F10 medium containing 5% fetal calf serum. The cells were restimulated the next day and were photographed 24 h later. (A) Cells not exposed to GMF; (B) cells exposed to GMF at 40 ng/mL, showing maximal response in process outgrowth; (C) as in (B) except that the GMF sample was adsorbed with monoclonal antibody against GMF; (D) as in (B) except that the GMF sample was adsorbed with conditioned medium from a hybridoma clone that did not produce anti-GMF antibody.

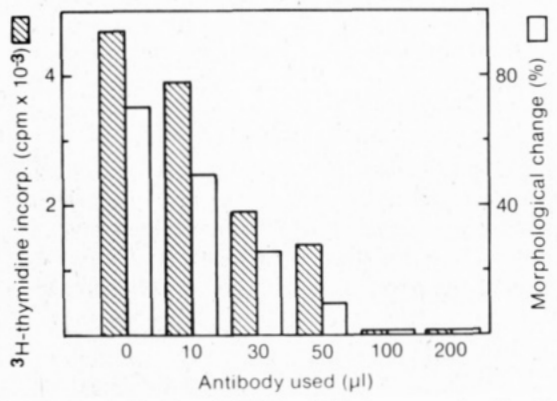


FIGURE 6: Neutralization of GMF activity by monoclonal antibody. Purified GMF was tested for mitogenic and morphological effects on confluent rat astroblasts after adsorption with various amounts of monoclonal antibody.

## Discussion

In this paper we demonstrated GMF as a single-chain, acidic polypeptide having a molecular weight of 14000. The identity of the purified product as GMF was verified by immunological means and by the recovery of activity from the protein band. The availability of pure GMF should now enable us to define more rigorously its function, to characterize the structure of the protein, and to study its molecular mechanism of action. The anti-GMF antibody will be a useful tool for determining its distribution and exploring its biological role.

Following our earliest reports, a number of other growth factors have been sighted in the brain or the pituitary. However, they differ from GMF either in chemical property or in target cell specificity, or both. The fibroblast growth factor (FGF) isolated from the pituitary is a basic protein having a molecular weight of 16000 (Gospodarowicz, 1975; Bohlen et al., 1984). It is a mitogen for fibroblasts and astrocytes but not Schwann cells (Gospodarowicz et al., 1978; Brockes et al., 1980). The glial growth factor from the pituitary is a 31000-dalton protein that shares target cell specificity with GMF. However, it differs from GMF not only in molecular weight but also in being a basic protein (Lemke & Brockes, 1984). The basic FGF from the brain is found to be identical with pituitary FGF (Gospodarowicz et al., 1984). The acidic FGF from the brain has a molecular weight of 16000 and an isoelectric point of pH 5.8, which are quite similar to those of GMF. However, it is a potent mitogen for endothelial cells derived from human umbilical vein (Lemmon et al., 1982; Thomas et al., 1984), whereas GMF is not (R. Lim and J. F. Miller, unpublished observations). Besides, acidic FGF has not been shown to be active for Schwann cells.

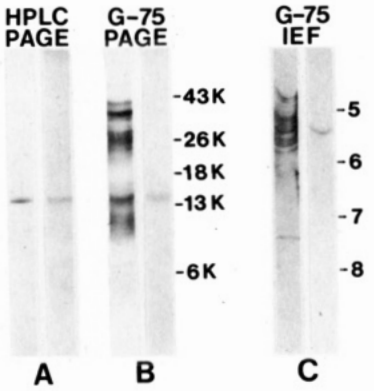


FIGURE 7: Binding of monoclonal antibody to the GMF band. (A) HPLC-purified GMF subjected to SDS–PAGE; (B) Sephadex G-75 partially purified GMF subjected to SDS–PAGE; (C) sample as in (B) subjected to IEF. Images on the left were gels stained with silver; those on the right were immunoblots. Molecular weights are indicated for SDS gels; pH values are indicated for IEF gel. SDS–PAGE was conducted with a 17% separation gel; IEF was with a pH range of 3.5–9.5.

Thus, the nervous tissue appears to be a major source of a family of growth or maturation factors with overlapping but not identical properties. Several of these factors are active against glial cells and thus may have autoregulatory functions in the brain. The study of their interaction may prove important in the understanding of the development, repair, and regeneration of the nervous system.

## Acknowledgments

We thank Bruce Baggenstoss for preparing the partially purified GMF and Dr. John E. Shively and David Hawke for performing the amino acid analysis.

## References

Bohlen, P., Baird, A., Esch, F., Ling, N., & Gospodarowicz, D. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 5364–5368.

Bosch, E. P., Assouline, J. G., Miller, J. F., & Lim, R. (1984) *Brain Res. 304*, 311–319.

Brockes, J. P., Lemke, G. E., & Balzer, D. A. (1980) *J. Biol. Chem. 255*, 8374–8377.

Cobbold, S. P., & Waldmann, H. (1981) *J. Immunol. Methods 44*, 125–133.

Fontana, A., Weber, E., Grob, P. J., Lim, R., & Miller, J. F. (1983) *J. Neuroimmunol. 5*, 261–269.

Gospodarowicz, D. (1975) *J. Biol. Chem. 250*, 2515–2520.

Gospodarowicz, D., Greenburg, G., Bialecki, H., & Zetter, B. (1978) *In Vitro 14*, 85–118.

Gospodarowicz, D., Cheng. J., Lui, G. M., Baird, A., & Bohlen, P. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 6963–6967.

Kennett, R. H. (1980) in *Monoclonal Antibodies* (Kennett, R. H., McKearn, T. J., & Bechtol, K. B., Eds.) pp 365–367, Plenum Press, New York.

Kessler, S. W. (1975) *J. Immunol. 115*, 1617–1624.
Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.
Lemke, G. E., & Brockes, J. P. (1984) *J. Neurosci. 4*, 75–83.
Lemmon, S. K., Riley, M. C., Thomas, K. A., Hoover, G. A., Maciag, T., & Bradshaw, R. A. (1982) *J. Cell Biol. 95*, 162–169.
Lim, R. (1980) *Curr. Top. Dev. Biol. 16*, 305–322.
Lim, R. (1985) in *Growth and Maturation Factors* (Guroff, G., Ed.) Vol. 3, pp 119–147, Wiley, New York.
Lim, R., & Mitsunobu, K. (1974) *Science (Washington, D.C.) 185*, 63–66.
Lim, R., & Miller, J. F. (1984) *J. Cell. Physiol. 119*, 255–259.
Lim, R., & Miller, J. F. (1985) *Experientia 41*, 412–415.
Lim, R., Li, W. K. P., & Mitsunobu, K. (1972) *Abstr. Annu. Meet. Soc. Neurosci., 2nd*, 181.
Lim, R., Mitsunobu, K., & Li, W. K. P. (1973) *Exp. Cell Res. 79*, 243–246.
Lim, R., Nakagawa, S., Arnason, B. G. W., & Turriff, D. E. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 4373–4377.
Miller, J. F., & Lim, R. (1984) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 43*, 2004.
Ogata, K., Arakawa, M., Kasahara, T., Shioiri-Nakano, K., & Hiraoka, K. (1983) *J. Immunol. Methods 65*, 75–82.
Sweadner, K. J. (1983) *J. Neurosci. 3*, 2505–2517.
Thomas, K. A., Rios-Candelore, M., & Fitzpatrick, S. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 357–361.
Towbin, H., Staehelin, T., & Gordon, J. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 4350–4354.

# Biosynthesis of Puromycin by *Streptomyces alboniger*: Characterization of Puromycin *N*-Acetyltransferase†

Jesus Vara, Jose A. Perez-Gonzalez, and Antonio Jimenez*
*Centro de Biología Molecular, UAM and CSIC, Universidad Autônoma de Madrid, Canto Blanco, Madrid 28049, Spain*
*Received June 10, 1985*

ABSTRACT: Puromycin *N*-acetyltransferase from *Streptomyces alboniger* inactivates puromycin by acetylating the amino position of its tyrosinyl moiety. This enzyme has been partially purified by column chromatography through DEAE-cellulose and Affigel Blue and characterized. It has an $M_r$ of 23 000, as determined by gel filtration. In addition to puromycin, the enzyme N-acetylates *O*-demethylpuromycin, a toxic precursor of the antibiotic, and chryscandin, a puromycin analogue antibiotic. The $K_m$ values for puromycin and *O*-demethylpuromycin are 1.7 and 4.6 μM, respectively. The *O*-demethylpuromycin *O*-methyltransferase from *S. alboniger*, which apparently catalyzes the last step in the biosynthesis of puromycin [Rao, M. M., Rebello, P. F., & Pogell, B. M. (1969) *J. Biol. Chem. 244*, 112–118], also O-methylates *N*-acetyl-*O*-demethylpuromycin. The $K_m$ values of the methylating enzyme for *O*-demethylpuromycin and *N*-acetyl-*O*-demethylpuromycin are 260 and 2.3 μM, respectively. These findings suggest that *O*-demethylpuromycin, if present in *S. alboniger*, would be N-acetylated and then O-methylated to be converted into *N*-acetylpuromycin. It might even be possible that N-acetylation of the puromycin backbone takes place at an earlier precursor.

The aminoacyl nucleoside antibiotic puromycin (Figure 1), which is produced by *Streptomyces alboniger*, is an inhibitor of both 70S and 80S ribosomes [for reviews, see Vazquez (1979) and Cundliffe (1981)]. Although ribosomes from *S. alboniger* are sensitive to puromycin, this bacterium contains an acetyltransferase that appears to inactivate the drug by acetylating its amino position (Pérez-González et al., 1983, 1985). Inactivation of antibiotics by acetylation or phosphorylation is a common process in aminocyclitol-producing *Streptomyces*. This type of drug modification has been implicated in the resistance of these bacteria to their own antibiotics products (Cella & Vining, 1975; Thompson et al., 1982; Matsuhashi et al., 1985). However, little is known about the role that the inactivating enzymes might play in the modification of antibiotic precursors. It is possible that at least some precursors are lethal for the producing organisms, being inactivated by the modifying enzymes [for a review, see Davies & Yagisawa (1983)]. *O*-Demethylpuromycin (Figure 1), the last precursor of puromycin biosynthesis (Rao et al., 1969), inhibits the ribosomes from *S. alboniger*, although at a lower extent than puromycin. *O*-Demethylpuromycin is acetylated by an enzymic activity that is present in cell-free extracts from this organism, the resulting *N*-acetyl-*O*-demethylpuromycin being inactive as tested in vitro (Pérez-González et al., 1985). These findings suggest that, in *S. alboniger*, puromycin may not be directly acetylated and the acetylation occurs at an earlier step in the biosynthetic pathway. To investigate this possibility, we have carried out a biochemical study of PAC. The results suggest that puromycin biosynthesis proceeds via *N*-acetyl-*O*-demethylpuromycin and that acetylation occurs on the *O*-demethylpuromycin intermediate or on an earlier intermediate.

## Materials and Methods

*Streptomyces Strains, Media, and Cell Growth. Streptomyces lividans* 1326, *S. alboniger* ATCC 12461, and *S. lividans* JN8 (carrying plasmid pFV8; Vara et al., 1985) were grown at 30 °C in either R2YE agar (Chater et al., 1982) or liquid YEME (Chater et al., 1982) supplemented with 34% (w/v) sucrose and 5 mM $MgCl_2$. Cultures were inoculated with spore suspensions that had been kept frozen in sterile 50% glycerol at −20 °C, according to Chater et al. (1982). Growth

† This research was supported by grants from the CAICYT (613/165) and the Plan concertado CAICYT-Antibióticos S.A. (631/051) and by an Institutional Grant from the Fondo de Investigaciones Sanitarias to the Centro de Biología Molecular. J. V. and J.A.P.-G. hold a fellowship from the Fondo de Investigaciones Sanitarias.

0006-2960/85/0424-8074$01.50/0 