Characterization of a Phosphotyrosyl Protein Phosphatase Activity Associated with a Phosphoseryl Protein Phosphatase of Mr = 95,000 from Bovine Heart*

(Received for publication, December 20, 1982)

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A cytosolic phosphoprotein phosphatase of Mr = 95,000 purified from bovine cardiac muscle, which contains a catalytic subunit of Mr = 35,000, is known to be associated with a Mg2+-activated p-nitrophenyl phosphatase activity. We have found that the enzyme preparation is also active toward phosphotyrosyl-IgG and -casein phosphorylated by pp60"'. The transform- ing gene product of Rous sarcoma virus. The properties of this phosphotyrosyl protein phosphatase activity closely resemble those of the p-nitrophenyl phospho- tase activity but sharply differ from those of the phos- phorylase phosphatase activity.

Comparative studies of the activities of the Mr = 95,000 phosphotyrosyl phosphatase, bovine kidney alkaline phosphatase, and ATP-Mg-dependent phosphatase toward phosphoseryl, phosphothreonyl, and phosphotyrosyl proteins and p-nitrophenyl phosphate under various conditions have been carried out. The results indicate that the Mr = 95,000 enzyme exhibits higher activity toward phosphoseryl and phosphothreonyl proteins than toward phosphotyrosyl proteins, while the kidney alkaline phosphatase preferentially dephosphorylates phosphotyrosyl proteins. ATP-Mg-dependent phosphatase is inactive toward phosphotyrosyl proteins.

Many sarcomagenic retroviruses recently have been shown to contain one gene coding for transformation-specific pro- teins which possess protein kinase activity (1–10). These protein kinases specifically phosphorylate tyrosine residues; a modification hitherto unknown (9, 11–13). It has been proposed that unregulated Tyr phosphorylation may be closely related to the establishment and maintenance of ma- lignant transformation in cells infected with RSV, as well as other retroviruses (1, 14). Normal cells also possess Tyr-specific protein kinase activity, although this activity is ex- pressed at much lower levels than in retrovirusally transformed cells (11, 14). This Tyr-protein kinase activity is highly con- served throughout evolution (15, 16), suggesting that phos- phorylation of Tyr residues in protein may be involved in certain essential cellular processes.

In previous communications (17–20), we have reported that a divalent cation-dependent PNP phosphatase activity is tightly associated with a phosphoseryl (P-Ser) protein phosphatase of Mr = 35,000 and its high molecular weight deriva- tives have been purified from cardiac muscle (17, 21) and several other tissues (18, 19). These two activities exhibit distinctly different catalytic properties. In this communication, we show that the cardiac Mr = 95,000 phosphoprotein phosphatase is active toward P-Tyr proteins and that the properties of this P-Tyr protein phosphatase activity resemble those of the PNP phosphatase and differ from those of the P-Ser protein phosphatase.

EXPERIMENTAL PROCEDURES

Materials

The catalytic subunit of cAMP-dependent protein kinase was purified from bovine heart according to Ref. 22. Inhibitor 1 was purified from rabbit skeletal muscle (23). Phosphorylase kinase and phosphorylase b (2X crystallized) were purchased from Sigma.

Antisera specific for pp60" were prepared by inducing tumors in newborn rabbits with RSV, Schmidt-Ruppin strain, group D, according to Ref. 24. For preparation of immunoadfinity resin specific for pp60"', IgG was partially purified from the antisera by 50% ammonium sulfate precipitation, followed by coupling to CNBr-activated Sepharose 4B (Pharmacia) according to Ref. 13. The pp60" was partially purified from a RSV-transformed rat cell line, AnAn (25), by the following procedures. AnAn cells were suspended in a buffer containing 10 mM KP, pH 7.0, 40% glycerol, 0.02% Nonidet P-40, 2 mM EDTA, 1 mM EGTA, 100 mM KC1, 2 mM 2-mercaptoethanol, and 1% aprotinin (Sigma). The cells were disrupted in a Dounce homogenizer and the suspension was centrifuged for 30 min at 30,000 × g. The supernatant was filtered through glass wool and applied to an aminomethyl agarose (Sigma) column equilibrated in Buffer A (5 mM KP, pH 7.0, 20% glycerol, 0.02% Nonidet P-40, 2 mM EDTA, 1 mM EGTA, 100 mM KC1, 2 mM 2-mercaptoethanol, and 1% aprotinin (Sigma)). The columns were disrupted by a Dounce homogenizer and the suspensions were centrifuged for 30 min at 30,000 × g. The supernatant was filtered through glass wool and applied to an aminomethyl agarose column equilibrated in Buffer A (5 mM KP, pH 7.0, 20% glycerol, 0.02% Nonidet P-40, 2 mM EDTA, 1 mM EGTA, 100 mM KC1, 2 mM 2-mercaptoethanol, and 1% aprotinin (Sigma)). The columns were disrupted by a Dounce homogenizer and the suspensions were centrifuged for 30 min at 30,000 × g.

*This work was supported by United States Public Health Service National Institutes of Health Grant HL-22662 and by American Cancer Society Grant CD22A. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 The abbreviations used are: RSV, Rous sarcoma virus; P-Tyr-, P-Ser-, P-Thr-protein, phosphoryseryl, phosphoryseryl, phosp- threonyl protein; PNP, p-nitrophenyl; PNP, p-nitrophenyl phosphatase; SDS, sodium dodecyl sulfate; EGTA, ethyleneglycol bis(ethyleneiminethylyl ether)-N,N,N',N'-tetraacetic acid; MES, 2-(N-morpho-
eluted by 2 column volumes of Buffer A plus 0.2 M KCl. The enzyme solution was immediately applied to an immunof affinity column. The column was washed with 5 volumes of Buffer A plus 1 M KCl and the pp60"-" was eluted with 2 column volumes of Buffer A plus 0.2 M KCl and 1.5 M KSCN. The enzyme was immediately dialyzed against Buffer A for 2 h followed by Buffer A plus 30% glycerol and stored at -20 °C. The pp60"-" so obtained is free from protein kinase activity which phosphorylates Ser or Thr residues.

Preparation of [32P]-Proteins

[32P]Tyr-IgG was prepared as follows. The pp60"-" in AnAn cells was immunocomplexed with the antisera and collected on protein A-Sepharose beads (Pharmacia). Phosphorylation of the heavy chain of IgG was carried out by incubating the protein A-Sepharose beads (20 mg Tris-HCl, pH 7.2, 5 mM MgCl2, and 1 mM dithiothreitol) with 20 mM Tris-HCl, pH 7.2, 5 mM MgCl2, and 1 mM dithiothreitol at 37 °C for 20 min. The protein A-Sepharose beads were then washed repeatedly with RIPA buffer (20 mM Tris-HCl, pH 7.2, 0.15 M NaCl, 1% Triton X-100, 0.1% SDS, and 1% deoxycholate) to remove unbound radioactivity. The [32P]-labeled complexes were separated from protein A-Sepharose by extracting with a small volume of 0.005 M HCl and dialyzed against 20 mM Tris-HCl, pH 7.2.

[32P]Tyr-casein was prepared by phosphorylation of α-casein (Sigma) with the partially purified pp60"-". The reaction was carried out at 20 °C for 30 min in an incubation mixture containing 0.1 M MES, pH 6.5, 1 mM MnCl2, 0.4 mM [γ-32P]ATP (3000 Ci/mmol), α-casein (1 mg/ml), and pp60"-" (70 μg/ml). The reaction was terminated by the addition of an equal volume of 25% trichloroacetic acid. The precipitated [32P]casein was extensively washed with 25% trichloroacetic acid and suspended in a small volume of distilled H2O. The suspension was then adjusted to pH 7.0 by 1 mM NaOH followed by extensive dialysis against 20 mM Tris-HCl, pH 7.2.

[32P]Thr-inhibitor 1 and [32P]-Ser-casein were prepared as follows. The phosphorylation reactions was carried out at 30 °C for 20 min in an incubation mixture containing 100 mM Tris-HCl, pH 7.0, 5 mM MgCl2, 0.5 unit/ml of the catalytic subunit of cAMP-dependent protein kinase, 0.4 mM [γ-32P]ATP (3000 Ci/mmol), 0.5 mg/ml of inhibitor 1 or 1 mg/ml of α-casein. The reactions were terminated and the [32P]-proteins were washed as described in the preceding paragraph.

[32P]-Ser-phosphorylase a of low specific radioactivity (0.15 Ci/mmol) was prepared as reported previously (25). [32P]-Phosphorylase a of high specific radioactivity (3000 Ci/mmol) was prepared by incubating crystalline phosphorylase b (1 mg/ml) with 50 mM Tris-HCl, pH 8.6, 5 mM MgCl2, 0.2 mM CaCl2, 0.4 mM [γ-32P]ATP (3000 Ci/mmol), and 1.2 units of phosphorylase kinase. After incubation at 30 °C for 30 min, 5 volumes of cold, saturating (NH4)2SO4 solution, pH 7.0, were added to the reaction mixture. [32P]-Phosphorylase a was collected by centrifugation at 4 °C, dissolved in a small volume of 20 mM Tris-HCl, pH 7.0, 10 mM 2-mercaptoethanol and reprecipitated by 5 volumes of the (NH4)2SO4 solution. This washing process was repeated five times and the [32P]-phosphorylase a obtained was stored at -20 °C or 37 °C in 0.1 M 2-mercaptoethanol and stored at 4 °C. Phosphoamino acid analysis of the [32P]-proteins shows that phosphorylation occurs exclusively at the specific amino acid residue as indicated.

Preparation of Phosphoprotein Phosphatases

Phosphoprotein phosphatase of M = 95,000 was purified from bovine cardiac muscle as previously described (20). The purification procedures include (NH4)2SO4 fractionation and DEAE-cellulose and Sephacryl S-200 chromatography. Evidence indicates that the enzyme contains a catalytic subunit of M = 35,000 (20). The phosphoprotein phosphatase of M = 35,000 was purified from rabbit liver by a procedure involving treatment of the enzyme with 80% ethanol as previously described (19). F1 and F2 were purified from bovine heart by procedures similar to those for purification of F1 and F2 from rabbit skeletal muscle (27, 28). Bovine kidney alkaline phosphatase was from Worthington.

Enzyme Assay—Phosphoprotein phosphatase assays were measured at 30 °C in an incubation volume of 25 μl, containing 50 mM Tris-HCl, pH 7.0 or 8.6, 1 mM dithiothreitol, 0.2-0.25 mM [32P]-protein (in terms of [32P]content), and 10 mM MgCl2. In experiments utilizing low specific activity phosphorylase a, phosphoprotein phosphatase activity was measured in an incubation volume of 25 μl, containing 50 mM Tris-HCl, pH 7.0, 1 mM dithiothreitol, 10 μM [32P]phosphorylase a, and 10 mM MgCl2. Low specific activity phosphorylase a was used for all studies unless otherwise indicated. Reactions were initiated by the addition of phosphatase and terminated by the addition of 5 μl of 25% trichloroacetic acid. Incubation time was adjusted such that no more than 20% of the substrate was dephosphorylated. The [32P] was released was separated from [32P]-protein by a paper chromatographic method as described previously (21). The amount of M = 95,000 enzyme used was about 20-fold greater when measuring P-Tyr-IgG phosphatase than phosphoprotein phosphatase activity.

p-Nitrophenyl phosphatase activity was measured by the release of p-nitrophenol from p-nitrophenol phosphate as described previously (17). The standard assay mixture (0.5 ml) contained 50 mM Tris-HCl, pH 8.6, 1 mM dithiothreitol, 20 mM PNPP, and 20 mM MgCl2. The reaction was initiated by the addition of enzyme, incubated at 30 °C for 30 min, and terminated by the addition of 0.5 ml of 1 M Na2CO3. The absorbance at 410 nm of the mixture was measured spectrophotometrically by using a control lacking enzyme as a blank (the extinction coefficient for p-nitrophenolate anion: 1.75 × 104 M-1 cm-1).

One unit of phosphatase activity was defined as the amount of enzyme catalyzing the release of 1 μmol of P/min for PNP phosphatase activity, and the release of 1 fmol of P/min for phosphoprotein phosphatase activity.

Polyacrylamide Gel Electrophoresis—Electrophoresis on 7% polyacrylamide gel was carried out by the procedure of Davis (29). Protein was stained with Coomassie brilliant blue. For localization phosphatase activity, gels were transversely sliced into 1-mm sections and each slice was placed in 100 μl of 50 mM Tris-HCl, pH 7.4, 10 mM 2-mercaptoethanol, 10 mM MgCl2, 50 mM KCl, and 10% glycerol for the extraction of the enzymatic activity. PNP phosphatase activity staining was done in 50 mM Tris-HCl, pH 8.6, 100 mM MgCl2, 20 mM PNPP, 1 mM dithiothreitol, and 0.2 M CaCl2. After incubation at 30 °C for a suitable length of time, a band of white calcium phosphate appeared on the gel, giving the location of the enzymatic activity.

Polyacrylamide gel electrophoresis in the presence of SDS was carried out according to Laemmli (30). [32P]-labeled proteins were located by autoradiography with the aid of Kodak Lanex intensifying screens.

Phosphoamino Acid Analysis—[32P]-labeled proteins were hydrolyzed in 6 N HCl for 2 h and subjected to thin layer electrophoresis at pH 3.5 according to the procedure of Hunter and Sefton (11).

Other Procedures—Protein concentration was determined by the method of Lowry et al. (31) following trichloroacetic acid precipitation. Bovine serum albumin was used as a standard.

RESULTS

Activation of P-Tyr-IgG and p-Nitrophenyl Phosphatase Activities by MgCl2—As shown in Fig. 1, the M = 95,000 phosphatase was activated by addition of Mg2+ to give a 30-fold increase in activity.

FIG. 1. Effects of MgCl2 on the activities of the cardiac phosphatase of M = 95,000. The enzymatic activities toward phosphorylase a (Phlase a) (C), P-Tyr-IgG (O), and p-nitrophenyl phosphate (×) were measured under standard assay conditions as described under "Experimental Procedures" except that MgCl2 concentrations were varied as indicated.
phosphatase preparation is active toward phosphorylase a in the absence of added divalent cation, but shows little, if any, activity toward either P-Tyr-IgG or PNPP. When Mg$^{2+}$ is added to the assay mixture, the enzymatic activity toward phosphorylase a is slightly inhibited, while those toward P-Tyr-IgG and PNPP become activated in a concentration-dependent manner. Both the saturation curves and the $K_m$ values for Mg$^{2+}$ (about 13 mM) for these two activities are similar.

**pH Optima**—Fig. 2 shows that the pH activity profiles of the P-Tyr-IgG and PNPP phosphatase activities are similar, and that both activities have an optimum around pH 8.5-9.0. In contrast, phosphorylase phosphatase activity, measured either in the presence of 2 mM EDTA (Fig. 2) or 20 mM MgCl$_2$ (data not shown), has an optimal pH around 7.5. It should be noted that, in the absence of Mg$^{2+}$, no activity toward P-Tyr-IgG (Fig. 2) or PNPP (data not shown) can be detected throughout the range of pH studied.

**Thermal Stability**—As shown in Fig. 3, the phosphorylase phosphatase activity is relatively stable at 40 °C. Both the P-Tyr-IgG and the PNPP phosphatase activities, however, are rapidly inactivated in a parallel fashion at this temperature. Within 30 min, the phosphatase becomes completely inactive toward these substrates.

**Effects of Phosphatase Inhibitors**—Fig. 4 shows the effects of increasing Pi concentrations on phosphorylase, P-Tyr-IgG, and PNPP phosphatase activities. Although all these activities are inhibited by millimolar levels of P$_i$, the phosphatase activities toward P-Tyr-IgG and PNPP are inhibited to a much greater extent than the phosphorylase phosphatase activity. Moreover, the degree of inhibition of P-Tyr-IgG and PNPP dephosphorylation is nearly identical at all levels of P$_i$ concentration. The concentrations of P$_i$, required for 50% inhibition of phosphatase activity is approximately 1 mM in the case of P-Tyr-IgG and PNPP and approximately 7 mM in the case of phosphorylase a.

Table I shows the effects of various compounds on the

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**Table I**

<table>
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<th>Addition</th>
<th>Cardiac phosphoprotein phosphatase</th>
<th>Kidney alkaline phosphatase</th>
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<td>Phosphorylase a</td>
<td>P-Tyr-IgG</td>
<td>P-Tyr-IgG</td>
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<tr>
<td>Phosphorylase a</td>
<td>PNPP</td>
<td>PNPP</td>
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<tr>
<td>50 μM ZnCl$_2$</td>
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<td>61 116 149</td>
</tr>
<tr>
<td>2 mM PNPP</td>
<td>100 34</td>
<td>24</td>
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<td>5 mM PNPP</td>
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<td>8</td>
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<td>50 mM NaF</td>
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<td>1 60 56</td>
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<td>5 mM EDTA</td>
<td>98 2.5</td>
<td>0 2.4 3.7</td>
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<td>2 mM PP</td>
<td>66 90 137</td>
<td>115 112</td>
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activities of the $M_s = 95,000$ phosphatase preparation from bovine cardiac muscle and of commercially obtained alkaline phosphatase from bovine kidney. The $M_s = 95,000$ preparation exhibits markedly different degrees of sensitivity to $Zn^{2+}$, EDTA, and $F^-$, whereas the phosphatase phosphatase activity is more sensitive in the presence of $50 \mu M Zn^{2+}$ and are inhibited about 40% in the presence of 50 mM $F^-$. By contrast, these two activities associated with the cardiac enzyme are inhibited about 30 and 99% in the presence of the same concentrations of $Zn^{2+}$ and $F^-$, respectively. Table I also shows that PNPP is a potent inhibitor of the P-Tyr-IgG phosphatase activity associated either with the cardiac or the kidney phosphatase. Furthermore, the P-Tyr-IgG phosphatase activity is more sensitive to inhibition by PNPP than the phosphatase phosphatase activity in the cardiac muscle enzyme preparation.

**Co-migration of the P-Tyr-IgG and the p-Nitrophenyl Phosphatase Activities on Polyacrylamide Gel Electrophoresis**—In previous communications, we have demonstrated that the PNP phosphatase activity co-purifies with the $M_s = 35,000$ phosphatase (17-19) and the $M_s = 95,000$ phosphatase which contains a $M_c = 35,000$ catalytic entity (20). We have examined the P-Tyr-IgG phosphatase activity in the processes of purification of the $M_s = 95,000$ phosphatase from bovine heart and the $M_s = 35,000$ enzyme from rabbit liver (32). The results indicate that the P-Tyr-phosphatase activity co-purifies with the PNP and the phosphatase phosphatase activities throughout various separation processes including ammonium sulfate fractionation, ethanol treatment, DEAE-cellulose and gel filtration chromatographies, and the polyacrylamide gel electrophoresis. Fig. 5 shows the results of polyacrylamide gel electrophoresis of a typical $M_s = 95,000$ phosphatase preparation. The data indicate that the enzymatic activities are separated into a single active peak of low mobility and doublet active peaks of high mobility. The enzymatic activity toward P-Tyr-IgG coincides with those toward PNPP and phosphorylase $\alpha$ in either the low mobility or the high mobility doublet bands. The activity profile shown in Fig. 5 reflects the fact that the highly purified $M_s = 95,000$ phosphatase tends to undergo partial dissociation on polyacrylamide gel electrophoresis (20). As previously reported (20), when the proteins in the low mobility band are extracted from the gel and re-electrophoresed on polyacrylamide in the presence of 50 mM $F^-$, the bands are observed. Similar experiments on the high mobility doublet active bands result in a single protein band of $M_s = 35,000$ on SDS-gel electrophoresis. It has been postulated that the $M_s = 95,000$ phosphatase consists of a catalytic subunit of $M_c = 35,000$ and a noncatalytic subunit of $M_s = 63,000$ (20). Regardless of the precise subunit composition of this enzyme, the present results clearly demonstrate that the activity toward P-Tyr-IgG is tightly associated with those toward PNPP and phosphorylase $\alpha$.

**Substrate Specificity**—In order to gain more understanding...
concerning the specificity of the phosphatase, $^{32}$P-proteins specifically labeled at Tyr, Ser, or Thr residues were used as substrates to study the enzymatic activity in various conditions. The concentrations of $^{32}$P-proteins used are all in the nanomolar range because of limited availability of $^{32}$P.)

Phosphatases were found to be sensitive to inhibition by EDTA, but were insensitive to F$^-$. Our findings concerning the properties of the activity of bovine kidney alkaline phosphatase toward various phosphoproteins are consistent with these data. Furthermore, our findings support the notion that various alkaline phosphatases, including cytosolic and membrane-bound enzymes, could function as P-Tyr-phosphatase in cells. It is interesting to note that F$_{33}$ which exhibits little PNP phosphatase activity, is inactive toward P-Tyr-proteins. Swarup et al. (33) have also reported that a P-Ser-phosphatase preparation from rabbit muscle shows some activity toward $^{32}$P-Tyr-histones. Whether this activity is similar to the P-Tyr-protein phosphatase described here is not clear.

Brautigan and co-workers have reported that a P-Tyr-protein phosphatase activity exists in membrane vesicles derived from A-431 cells (35) and RSV-transformed rat cells (36). This activity was slightly stimulated by EDTA and F$^-$, but was strongly inhibited by micromolar Zn$^{2+}$. An EDTA-insensitive, Zn$^{2+}$-sensitive P-Tyr-protein phosphatase activity has also been found in crude extracts of rat liver and muscle (37). The properties of these enzymatic activities appear to be different from those of P-Tyr-protein phosphatases described in this report.

The present studies indicate that the P-Tyr-protein phosphatase activity is much lower than the P-Ser-protein phosphatase activity in the $M_e = 95,000$ enzyme preparation, when the enzymatic activities are measured with phosphoproteins in nanomolar concentrations (Table I). Furthermore, the ratio of the P-Tyr-protein phosphatase to the PNP phosphatase activity of the $M_e = 95,000$ enzyme preparation is lower than that of the kidney alkaline phosphatase. These data seem to indicate that the P-Tyr-protein phosphatase activity in the $M_e = 95,000$ preparation represents a minor activity in animal tissues. However, since the physiological substrate(s) for pp60$^{+}$ or other P-Tyr-protein kinases is not known, the possibility that this phosphatase activity, as detected using $^{32}$P-Tyr-IgG and $^{32}$P-Tyr-caseins as substrates, might participate in the phosphoprotein activity of certain biologically important P-Tyr-proteins cannot be ruled out. It is also possible that it represents a latent form of the enzyme which requires certain unknown metabolite(s) for activation. The observation that the cardiac and the kidney phosphatases preferentially dephosphorylate $^{32}$P-Tyr-casein and $^{32}$P-Tyr-IgG, respectively (Table II), indicates that in animal tissues there exist different P-Tyr-protein phosphatases with distinct substrate specificities. They may play different roles in the reg-
ulation of the dephosphorylation of cellular P-Tyr-proteins. The fact that a P-Tyr-protein phosphatase activity closely associates with a P-Ser-protein phosphatase activity is intriguing. Many P-Tyr-proteins, including pp60<sup>src</sup> itself, have been shown to contain in addition P-Ser and/or P-Thr residues (1, 21). It is tempting to speculate that the close association of these two different phosphatase activities may represent a device for the coordinate dephosphorylation of P-Tyr and P-Ser and/or P-Thr residues in a given protein.

Acknowledgment—We wish to thank Wanda W. S. Chan for her excellent technical assistance.

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