Biochemical Pharmacology, Vol. 28, pp. 1519–1523.
Pergamon Press Ltd., 1979. Printed in Great Britain.

COVALENT BINDING OF PROSTAGLANDINS G_2 AND H_2 TO COMPONENTS OF RAM SEMINAL VESICLE MICROSOMAL FRACTION

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(*Received* 7 *August* 1978; *accepted* 7 *November* 1978)

Abstract—Following incubation of [1-^{14}C]arachidonic acid with a microsomal preparation of ram seminal vesicles, 90 per cent of the products formed were classical prostaglandins and hydroxyfatty acid. In addition, approximately 8 per cent of the products formed possessed radioactivity which was covalently bound to the microsomal pellet after acid precipitation and solvent extraction. The amount of covalently bound material derived from arachidonate was related to the concentration of free prostaglandin (PG) endoperoxides in the incubation mixture, indicating that these reactive intermediates were probably responsible for the generation of the covalently bound material. Protein-associated radioactivity was also observed following incubation of [1-^{14}C]PGG_2 or [1-^{14}C]PGH_2 with a heat-denatured preparation of ram seminal vesicle microsomes. Such preparations do not convert PGG_2 to PGH_2; thus, the binding of PGG_2 was not due to its prior conversion to PGH_2. The rate constant for the covalent binding of PGG_2 (0.19 min^{-1}) was approximately twice that for PGH_2 (0.11 min^{-1}), suggesting that both 9,11-endoperoxy and 15-hydroperoxy groups participate in the binding reaction. Moreover, the rate constant of binding derived from arachidonate (0.12 min^{-1}) approximated those of the purified endoperoxides. In an active microsomal preparation, glutathione reduced covalent binding and increased PGE_2 formation without greatly affecting oxygen consumption and, therefore, without affecting total product formation from arachidonic acid. Glutathione, therefore, probably inhibited covalent binding by lowering free endoperoxide concentrations in the incubation mixture. Diethyldithiocarbamate and phenylbutazone, but not para-aminophenol or butylated hydroxyanisole, inhibited the covalent binding of PGG_2 to heat-denatured tissue protein. These results suggest that the covalent binding reaction may proceed via the formation of free radicals.

Previous work has demonstrated that incubation of [1-^{14}C]arachidonic acid with the microsomal fraction of guinea pig lung led to the covalent binding of a small but significant proportion of radioactive material to the microsomal protein [1]. This was inhibited by indomethacin, suggesting that the material was derived via the cyclo-oxygenase pathway. Covalently bound material was also formed when [1-^{14}C]arachidonic acid was incubated with the microsomal fraction of several other cyclo-oxygenase-containing tissues, including ram and bovine seminal vesicles, human platelets, rabbit kidney and rat fundus [2], suggesting that the covalently bound material was derived from an intermediate common to the prostaglandin (PG) biosynthesis in each tissue. The prostaglandin endoperoxides, PGG_2 and PGH_2 play a pivotal role in the biosynthesis of classical prostaglandins [3, 4], prostacyclin [5] and thromboxanes [6]. They are also chemically reactive, as shown by their relatively short half-lives in aqueous media [7]. The prostaglandin endoperoxides, therefore, would seem to be primary candidates for the role of active intermediates from which covalently bound material is derived.

A variety of carcinogenic or mutagenic substances exert their toxic effects via conversion, usually by the mixed-function oxidases, to reactive intermediates which bind covalently to tissue macromolecules [8–10]. Covalent binding of electrophilic metabolites to cellular protein also precedes the bronchiolar and hepatic necrosis induced by halogenated aryl hydrocarbons [11, 12]. The finding that the cyclo-oxygenase system also generates intermediates capable of covalent binding may, therefore, be of pathological significance. The present studies further investigate the covalent binding derived from [1-^{14}C]arachidonic acid in ram seminal vesicle microsomes and provide evidence that the prostaglandin endoperoxides do indeed bind covalently to tissue macromolecules.

EXPERIMENTAL

Materials

Arachidonic acid was obtained from Nu Chek Prep., Inc., Elysian, MN, and [1-^{14}C]arachidonic acid, specific activity 60 mCi/m-mole, from the Amersham Corp., Arlington Heights, IL. The following tritium-labeled compounds were obtained from New England Nuclear, Boston, MA, and were used as standards for thin-layer chromatography: [5,6,8,9,11,12,14,15-^{3}H]arachidonic acid, [5,6,8,11,13,14,15-^{3}H]PGE_2, [9-^{3}H]$PGF_{2\alpha}$ and [5,6-^{3}H]PGB_1. Glutathione, L-tryptophan, butylated hydroxyanisole and phenylbutazone were purchased from the Sigma Chemical Co., St. Louis, MO. Triphenylphosphine was purchased from the Aldrich Chemical Co., Milwaukee, WI. Para-aminophenol was purchased from the Eastman Kodak Co., Rochester, NY. Silica gel G thin-layer chromatography plates, 250 μm, were purchased from Analtech, Inc., Newark, DE. Silicic acid, 100 mesh, was obtained from Mallinckrodt, St. Louis, MO. Aquasol scintillation

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mixture was obtained from New England Nuclear, Boston, MA.

Methods

Incubation procedure. The microsomal fraction of ram seminal vesicles was prepared as described previously [13]. All incubations were carried out at 22–24°. Incubation mixtures had the following composition: microsomal protein, 2 mg/ml; sucrose, 125 mM; HEPES buffer, pH 7.5, 125 μM; [1-^{14}C]arachidonic acid, 90 μM, 1.3×10^6 dis./min/ml. In some experiments, reduced glutathione was included in the incubation mixture prior to the addition of substrate. In experiments in which prostaglandin endoperoxides were used, the microsomal suspension was denatured by boiling for 30 min before incorporation into the incubation mixture and the addition of [1-^{14}C]PGG_2, 1.8 μM, 1×10^5 dis./min/ml, or [1-^{14}C]PGH_2, 0.8 μM, 5×10^4 dis./min/ml. In some experiments, antioxidants were added to the mixtures 5 min prior to the addition of [1-^{14}C]PGG_2.

Oxygen consumption studies. Oxygen consumption studies were carried out in a Clark oxygen electrode maintained at 24°. Incubation mixtures consisted of 2 ml of the mixture described. The reaction was stopped at suitable times after the addition of substrate by the removal of 1 ml incubate, which was then examined for covalently bound material as described below.

Measurement of free endoperoxide concentration. Following initiation of the reaction by the addition of the appropriate substrate, 2×1 ml samples were simultaneously withdrawn at suitable times. One sample was added to 6 ml ethyl acetate containing 0.2%, v/v, formic acid. The second sample was added to 6 ml ethyl acetate containing formic acid and 6 mg triphenylphosphine, which reduced free endoperoxides to $PGF_{2\alpha}$. Following vortexing and phase separation, the aqueous layers were further extracted thrice with 6 ml ethyl acetate. The organic phases were pooled, reduced to dryness *in vacuo*, and the residues were examined by thin-layer chromatography in the solvent system chloroform–methanol–acetic acid–water (90:8:1:0.8) v/v [4]. Authentic tritium-labeled arachidonic acid and prostaglandins E_2 and $F_{2\alpha}$ were applied to the plates as standards. After development, the plates were scraped in 0.4-cm sections from the origin to the solvent front, and the scrapings were mixed with 10 ml Aquasol scintillation mixture. Radioactivity in each section was determined by liquid scintillation counting.

Measurement of covalently bound radioactivity. This was performed by a method described previously [1]. The protein in the aqueous layers following ethyl acetate extraction was precipitated and washed with 10% trichloroacetic acid. The protein pellet was extracted with chloroform–methanol (2:1, v/v) until radioactivity could not be detected in the washings. The pellet was similarly extracted with methanol–water (4:1, v/v) and then solubilized in 1 N NaOH. Protein was estimated by the method of Lowry *et al.* [14]. One-ml aliquots were mixed with 1 ml water and 15 ml Aquasol scintillation mixture and radioactivity was measured by liquid scintillation techniques. Radioactivity associated with the pellet after solvent extraction was termed "covalently bound material".

Preparation of prostaglandin endoperoxides. [1-^{14}C] prostaglandins G_2 and H_2 were prepared essentially according to published procedures [7], and were purified by chromatography on silicic acid −5°. PGG_2 was eluted with ether–hexane (2:3, v/v) and PGH_2 was eluted with ether–hexane (3:2, v/v). The identity of the endoperoxides was established by the following procedures: thin-layer chromatography at −5° in the solvent system ether–petroleum ether–acetic acid (85:15:0.1, v/v) [15], by reduction with triphenylphosphine or stannous chloride [7], and by degradation in aqueous medium [7]. The endoperoxides were found to be 70–90 per cent pure by these methods.

RESULTS

Arachidonic acid, 90 μM, was incubated with the microsomal fraction of ram seminal vesicles. After acid lipid extraction and in the absence of triphenylphosphine in the incubation mixture, examination by thin layer chromatography revealed unchanged arachidonate together with PGE_2, lesser amounts of PGD_2 and $PGF_{2\alpha}$, and an unidentified hydroxyfatty acid, which was probably 12-hydroxy-5, 8, 10-heptadecatrienoic acid (HHT). No PGA_2 was detected. This is in agreement with previous reports [3, 16]. Prostaglandins and HHT formed 90 per cent of the products derived from arachidonate. In addition, approximately 8 per cent of radioactive products derived from arachidonate were not removed by acid lipid extraction but remained associated material was considered to be covalently tation and solvent extraction. This macromolecule associated materials was considered to be covalently bound [1]. Approximately 2 per cent of products formed were detected in the trichloroacetic acid washings, and were possibly water-soluble glutathione conjugates [1].

Correlation between covalent binding and estimated free endoperoxide levels

If the hypothesis is correct that the covalently bound material is derived from prostaglandin endoperoxides, and that the binding follows simple second-order kinetics, then

$$\frac{d}{dt}\,(\text{covalent binding}) = k\,[\text{endoperoxide}]\,[\text{protein}]$$

If the receptor protein is assumed to be in excess, then the [protein] remains essentially constant, and

$$\frac{d}{dt}\,(\text{covalent binding}) = k'\,[\text{endoperoxide}]$$

Thus, covalant binding at time T after initiation of the reaction is equal to:

$$\begin{matrix}\text{Covalent}\\ \text{Binding}\end{matrix} = k' \int_0^T [\text{endoperoxide}] \cdot dt$$

The relationship between covalent binding and the area under the free endoperoxide concentration curve should thus be linear.

Following incubation of [1-^{14}C] arachidonic acid with ram seminal vesicle microsomes, samples were withdrawn and the free endoperoxides were reduced to $PGF_{2\alpha}$ by triphenylphosphine. The time-course of $PGF_{2\alpha}$ formation in both reduced and nonreduced samples is shown in Fig. 1. $PGF_{2\alpha}$ levels were higher in reduced samples; these were corrected by subtraction of $PGF_{2\alpha}$ in the appropriate nonreduced samples. The corrected time-course reached a peak at approximately

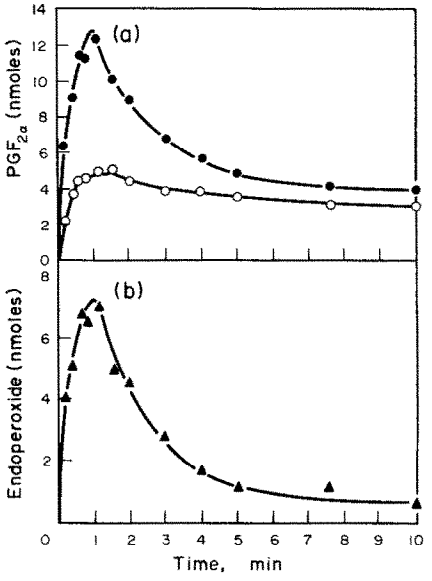

Fig. 1. Upper panel: time-course of $PGF_{2\alpha}$ formation following incubation of [1-^{14}C]arachidonic acid with ram seminal vesicle microsomes. At suitable times after starting incubation, duplicate 1-ml samples were simultaneously withdrawn and added to either a reducing extraction mixture consisting of ethyl acetate, formic acid and triphenylphosphine, or a non-reducing extraction mixture consisting of ethyl acetate and formic acid. After extraction, $PGF_{2\alpha}$ was measured by thin-layer chromatography. Key: (●——●) $PGF_{2\alpha}$ in reduced samples; and (○——○) $PGF_{2\alpha}$ in non-reduced samples. Lower panel: time-course of estimated levels of free endoperoxides. $PGF_{2\alpha}$ in reduced samples was corrected by subtraction of $PGF_{2\alpha}$ in non-reduced samples, and also corrected for reduction efficiency.

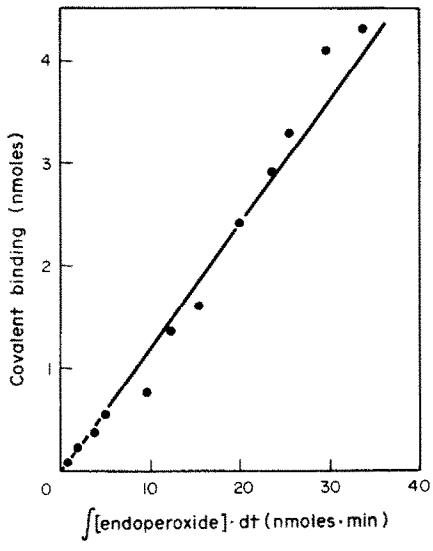

Fig. 2. Correlation between covalent binding and total endoperoxide formation following incubation of [1-^{14}C]arachidonic acid with ram seminal vesicle microsomes. The area under the free endoperoxide curve was estimated at suitable time increments and correlated with covalent binding at these times. Line was fitted by least squares regression analysis. Correlation coefficient, −0.958.

1 min and declined by 10 min (Fig. 1b). This is consistent with the time-course of endoperoxide formation from 8, 11, 14-eicosatrienoic acid by ram seminal vesicle microsomes at 28° [4]; hence, the time-course probably accurately reflects free endoperoxide levels. The efficiency of reduction using purified prostaglandin endoperoxides in both active and boiled microsomes was 70 per cent for both PGG_2 and PGH_2 and was independent of the endoperoxide concentration over the range studied. $PGF_{2\alpha}$ levels were converted to enderpoxide levels assuming a reduction efficiency of 70 per cent, and the area under the resulting curve was estimated and correlated with covalently bound material. The relationship was linear (Fig. 2), and, therefore, is consistent with the hypothesis that the covalently bound material is derived from PGG_2, PGH_2 or both endoperoxides. The pseudo-first-order rate constant for the covalent binding was estimated from the slope of the line and found to be 0.12 min^{-1}.

Covalent binding of prostaglandins G_2 and H_2. In order to determine which endoperoxide was responsible for the generation of covalently bound material, [1-^{14}C]prostaglandins G_2 and H_2 were incubated with ram seminal vesicle microsomes. Heat-denatured microsomes were used to facilitate the measurement of endoperoxides in solution by preventing their rapid conversion to end products. Heat-denaturation also prevented the enzymic conversion of PGG_2 to PGH_2 [15], and thus allowed determination of the direct effects of PGG_2. Covalently bound radioactivity was observed following incubation of either PGG_2 or PGH_2 with the heat-denatured microsomal fraction.

Free endoperoxides were estimated, and the areas under their decay curves were measured and correlated with covalently bound meterial. The relationships between area and amount bound were linear (Fig. 3), with

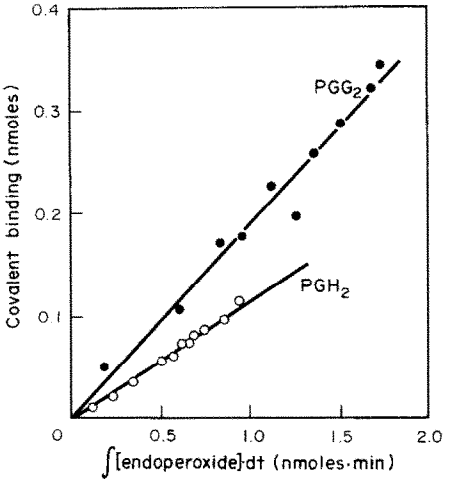

Fig. 3. Correlation between covalent binding and total endoperoxide concentration following incubation of [1-^{14}C]prostaglandins G_2 or H_2 with boiled ram seminal vesicle microsomes. Free concentrations of PGG_2 and PGH_2 were measured as described, and their areas at suitable time increments were estimated and correlated with covalent binding at these times. Key: (●——●) PGG_2; and (○——○) PGH_2. Lines were fitted by least squares regression analysis. Correlation coefficient = 0.958 and 0.997 respectively.

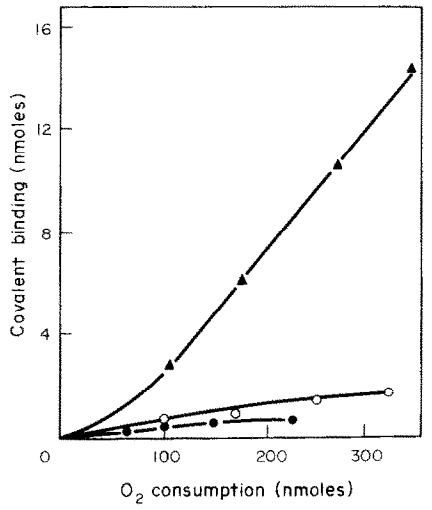

Fig. 4. Effect of glutathione on covalent binding following incubation of [1-^{14}C]arachidonic acid with ram seminal vesicle microsomes. Incubations were carried out in a Clark oxygen electrode. At suitable times after addition of arachidonate, the incubation was stopped by the withdrawal of 1-ml samples. Covalent binding was estimated after precipitation and exhaustive solvent extraction of the protein. The degree of covalently bound material was correlated with total oxygen consumed by the reaction. Suitable amounts of glutathione were added to the incubation mixture immediately prior to the addition of substrate. Key: (▲——▲) control; (●——●) glutathione, 0.5 mM; (○——○) glutathione, 5.0 mM.

correlation coefficients of 0.985 and 0.997 respectively. The rate constant of binding of PGG_2, assuming a reduction efficiency of 70 per cent, was estimated to be 0.19 min^{-1}, while that for PGH_2 was 0.11 min^{-1}.

Inhibition of covalent binding. Studies with glutathione were carried out in a Clark oxygen electrode. [1-^{14}C] arachidonic acid was incubated with ram seminal vesicle microsomes in the presence or absence of suitable amounts of reduced glutathione. Samples were withdrawn at intervals and the covalently bound material was determined. Correlation between covalent binding and oxygen consumption is shown in Fig. 4. In experiments in which stable prostaglandins were also estimated by thin-layer chromatography, and water-soluble metabolites were quantified, we confirmed previous reports [17] that 2 moles of oxygen are consumed per 1 mole of total product formed. Glutathione markedly inhibited the generation of covalently bound material without greatly affecting oxygen consumption, and, therefore, total product formation. In addition, the sole acid–lipid extractable product derived from arachidonate in the presence of glutathione was PGE_2.

The effect of several antioxidants on the covalent binding of [1-^{14}C]PGG_2 to boiled microsomal protein was studied. Both diethyldithiocarbamate and phenylbutazone inhibited the binding. Para-aminophenol and butylated hydroxyanisole were inactive at the concentrations used (Table 1).

DISCUSSION

Incubation of [1-^{14}C]arachidonic acid with a microsomal preparation of ram seminal vesicles led to the formation of macromolecule-associated radioactivity which could not be dissociated by the solvent extraction procedures employed. Similar protein-associated material formed following incubation of [1-^{14}C]arachidonic acid with guinea pig lung microsomes was not dissociated after digestion by guanidine-HCl followed by Sephadex chromatography [1] or by refluxing with methanol for up to 90 min.* Covalently bound radioactivity was reduced following incubation of [1-^{14}C]arachidonate with guinea pig lung microsomes in the presence of indomethacin and was not observed on incubation with heat-denatured microsomes [1]; hence, the binding is not due to fatty acid association with albumin. Tritium-labeled PG E_2, $F_{2\alpha}$ and A_2 did not

* A. G. E. Wilson, personal communication.

Table 1. Effects of antioxidants on covalent binding of [1-^{14}C]PGG_2 to heat-denatured ram seminal vesicle microsomal protein*

Compound	Concn (mM)	Inhibition of covalent binding (%)
Diethyldithiocarbamate	1	32
	5	52
Phenylbutazone	1	16
	10	62
Para-aminophenol	1	0
	10	0
Butylated hydroxyanisole	5	0

* Incubation mixtures containing boiled microsomes, as described in Methods, were incubated at 22–24° for 5 min in the presence of suitable concentrations of antioxidants. [1-^{14}C]PGG_2, 1 nmole/ml, was added and incubated for 8 min. Covalently bound material was measured after protein precipitation and exhaustive solvent extraction. Inhibition values quoted are the mean of five determinations (diethyldithiocarbamate and phenylbutazone) or of two determinations (para-aminophenol and butylated hydroxyanisole). In the absence of antioxidants, covalently bound material was 74 ± 7 pmoles/nmole of PGG_2 added (mean ± S. E. M., thirteen determinations).

covalently associate with guinea pig lung microsomal fraction [1]; this is to be expected since their structures would make covalent binding to protein unlikely. These findings when considered together strongly suggest that the protein-associated material derived from [1-^{14}C]arachidonic acid following incubation with active ram seminal vesicles was covalently bound [1, 11].

The present studies show that the covalent binding observed when [1-^{14}C]arachidonic acid was incubated with ram seminal vesicle microsomes can probably be attributed to the prostaglandin endoperoxides PGG_2 and PGH_2, since the degree of binding at any time was related to the free endoperoxide concentration. Moreover, the rate constant of binding derived from arachidonic acid approximated those of the endoperoxides themselves. The binding of PGG_2 cannot be ascribed to its enzymatic conversion to PGH_2 since binding of both purified prostaglandins occurred in heat-denatured tissue. The rate constant for the covalent binding of PGG_2 (0.19 min^{-1}) was approximately twice that for PGH_2 (0.11 min^{-1}), presumably indicating that the 9, 11-endoperoxy group of PGH_2 and both the 9, 11-endoperoxy and the 15-hydroperoxy groups of PGG_2 participate in the binding reaction.

Glutathione reduced covalent binding more than it affected total product formation from [1-^{14}C]arachidonate. Glutathione is an absolute requirement for prostaglandin E isomerase, and stimulates the conversion of PGH_2 to PGE_2 [18]. This is confirmed by the present studies since in the presence of glutathione the sole acid-lipid product was PGE_2.

Prostaglandins D_2 and F_2 and HHT formed in the absence of glutathione may have been due to non-enzymic breakdown of the endoperoxides during the incubation of work-up procedures. The stimulation of PGE_2 formation, therefore, would lower free endoperoxide levels [2]. Glutathione may also lower free endoperoxide concentrations by the formation of water-soluble adducts [1]. Lowering of free endoperoxide levels probably accounts for the inhibiting action of glutathione on covalent binding.

The mechanism by which the binding takes place is not known at present. However, the formation of adducts between protein and lipid peroxide generally proceeds via the formation of free radicals [19]. Prostaglandin endoperoxides may covalently bind to protein by a similar type of mechanism. Such a mechanism is consistent with the finding that the binding of PGG_2 to protein was inhibited by diethyldithiocarbamate and by phenylbutazone. Diethyldithiocarbamate inhibits free radical propagation [20]; phenylbutazone, which contains an enolic hydrogen, may also act as a free radical scavenger. However, relatively high concentrations of these compounds were required to inhibit the binding; in addition, the phenolic antioxidants para-aminophenol and butylated hydroxyanisole were inactive. Alternatively, the binding may proceed via interaction of the endoperoxides with nucleophilic sites on the protein in a manner similar to that of electrophilic oxides produced from a variety of xenobiotics by the mixed-function oxidases [8–10]. The possibility that the binding may proceed via free radical formation has important implications since the widespread tissue damage produced by such reactive species even at low concentrations is well documented [19].

The specific protein, nucleic acid or other residue to which the endoperoxides covalently bind is also unknown at present. It is possible that binding occurs to the cyclo-oxygenase itself, and may play a role in the regulation of its activity. It is known that cyclo-oxygenase undergoes self-inactivation; Egan *et al.* [17] have suggested that this is due to the release of an oxygen-centered radical during conversion of PGG_2 to PGH_2, and, in support of this, have shown that PGG_2, but not PGH_2, inhibits cyclo-oxygenase. However, Marnett *et al.* [21] have provided evidence that both PGG_2 and PGH_2 inhibit cyclo-oxygenase. The possibility of covalent binding of prostaglandin endoperoxides to cyclo-oxygenase deserves further investigation.

Acknowledgements—We wish to thank Mr. Jeff Boyd and Ms. Mary Jo Hicks for excellent technical assistance, and Ms. Denise Crawford for help in the preparation of the manuscript.

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