PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE **183**, 118–124 (1986)

Glycosylation in Livers of Newborn Mice Homozygous for a Lethal Deletion (42395)

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Abstract. Endogenous glycoprotein and lipid biosynthesis have been examined in slices of liver and other organs from normal and mutant mice homozygous for a perinatally lethal deletion in chromosome 7. Pronase digests of total glycoproteins, radioactively labeled with glucosamine, followed by Bio-Gel P-6 column chromatography of the resultant glycopeptides, indicate that glycosylation in mutant mouse liver is dramatically reduced compared to that of normal littermates. Pulse–chase experiments suggest that this reduction is not due to a processing event, but rather to reduced biosynthesis. In addition, a quantitative reduction of glycopeptides was observed in mutant livers, when the radioactive peaks from the Bio-Gel P-6 fractionation were pooled and analyzed on a Dowex 50 column, followed by separation on DE-52 columns. Analysis, by affinity chromatography, of radioactively labeled total lipids indicated that homozygous mutant and normal littermate livers have similar quantities of neutral and acidic lipids, including phosphatidylserine, phosphatidylinositol, cerebrosides, and phospholipids. Furthermore, the analysis of other organs indicates that the reduction of glycoprotein synthesis observed in the mutant liver is specific to this organ.

Several specific membrane and serum protein abnormalities have been reported in livers of newborn mice homozygous for overlapping perinatally lethal deletions mapping near the albino (*c*) locus of chromosome 7 (1). Electron microscopic studies demonstrated ultrastructural abnormalities, including vesiculation and dilation of the rough endoplasmic reticulum (RER), the Golgi apparatus and the nuclear membrane (2). Three serum proteins (α-fetoprotein, transferrin, and albumin), two of them glycoproteins, were found to be reduced in quantity (3). In addition, more recent studies revealed a 75% reduction in number of three plasma membrane bound receptors (4, 5). These data, as well as the fact that both membrane-associated and secretory glycoproteins are synthesized in the RER and processed in the Golgi complex, both of them abnormal in these mutants (6), prompted an investigation of glycosylation in the livers of mice homozygous for lethal deletions in chromosome 7.

Materials and Methods. *Normal and mutant mice.* Mice carrying the lethal albino deletions c^{3H}, c^{14CoS}, and c^{112K} were bred as heterozygotes with c^{ch} (chinchilla) as the normal allele. Mutant homozygous newborn mice were recognized as albinos by their unpigmented eyes; the heterozygous c^{ch}/c^{3H} newborns could be distinguished from homozygous normal newborns by dilution of eye pigmentation. Animals were sacrificed by decapitation within a few hours after spontaneous birth, and the organs were removed, weighed, and sliced.

Incorporation of [6-^{3}H]glucosamine into endogenous proteins and lipids. Organs were sliced (1-mm slices) and placed in a petri dish with 0.5 ml Hanks' balanced salt solution (BSS) containing Ca^{2+} and Mg^{2+} (GIBCO) or in 0.5 ml Dulbecco's modified essential medium [(MEM) GIBCO]. The tissue suspensions were transferred to test tubes (18 × 150 mm) containing 40 μCi [6-^{3}H]glucosamine hydrochloride (Amersham, 24.8 Ci/mmole) in dry form. The petri dishes were rinsed once with 0.5 ml Hanks' BSS or Dulbecco's MEM which was added to its respective tube (final volume was 1 ml). The slice suspensions were

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0037-9727/86 $1.50

gassed with O_2 and incubated at 37°C for specific time intervals. The reaction was terminated by the addition of 5 ml of chloroform and methanol (3:2) to give a final ratio of chloroform:methanol:water of 3:2:1.

Extraction of total lipids and glycoproteins. Total lipids and glycoproteins were subjected to sequential extractions with chloroform: methanol:water (C:M:W, 3:2:1) as described by Adamany and Spiro (7). Dolichylsaccharides were extracted with C:M:W (10:10:3) according to Parodi *et al.* (8). Briefly, the 6-ml tissue suspensions containing C:M:W at 3:2:1 were vortexed for 30 min and centrifuged at 2000 rpm in a Sorvall GLC-2 centrifuge to obtain: (i) an upper phase, containing nucleotide sugars and other soluble components, (ii) a lower phase containing total lipids (sterols, phospholipids, and other cellular lipids), and (iii) an interphase containing total glycoproteins. The interphase was extracted three additional times with 6 ml C:M:W (3:2:1), once for 30 min and twice for 10 min. The upper phases were pooled and the combined lower phases were washed three times with 8 ml of C:M:W (1:16:16). The interphase was extracted three times with 6 ml of C:M:W (10:10:3) to obtain the dolichylsaccharides, leaving a proteinaceous residue of cellular glycoproteins. The radioactivity of the combined extracts was determined by scintillation counting.

Digestion of cellular glycoproteins. Glycopeptides were prepared by incubation at 37°C of the dried delipidated proteinaceous residue for 5 days with 6 mg/ml of Pronase (Calbiochem, 50 TU) in 3 ml of 0.15 *M* Tris-acetate (pH 7.8) containing 0.001 *M* $CaCl_2$ under toluene. Fresh Pronase solution (0.1 ml of 6 mg/ml) was added daily. The samples were dried to 0.5 ml using an Evapo-Mix (Buchler Instruments), and 1 ml of deionized H_2O was added to each sample.

Separation of radioactively labeled glycopeptides. The Pronase digests were fractionated on a column (1.8 × 85.7 cm) of Bio-Gel P-6 previously equilibrated with 0.1 *M* pyridine acetate buffer, pH 5.0. Elution of glycopeptides was achieved with the same buffer; 60 fractions containing 2.5 ml each were collected. Aliquots (0.2 ml) of each fraction were taken for liquid scintillation counting (Liquiscint, General Diagnostics) in a Packard Tri-Carb spectrometer.

Fractionation of labeled glycopeptides obtained from Bio-Gel P-6 chromatography. The radioactively labeled glycopeptides from Bio-Gel P-6 chromatography were pooled and designated as peaks 1, 2, and 3. Peaks 2 and 3 were subjected to fractionation on a Dowex 50 × 2 (200–400) column (1.4 × 24 cm) previously washed with 2 *M* pyridine, 0.2 *M* pyridine formate (pH 3), and equilibrated with 1 m*M* pyridine formate (pH 3). The sample was loaded onto the column and 16 tubes (2.5 ml per fraction) were eluted with 1 m*M* pyridine formate (pH 3), followed by a gradient (125 ml each) of 1 m*M* pyridine (pH 3) to 0.3 *M* pyridine formate (pH 3). Four-tenths of a milliliter of each fraction was counted for radioactivity.

Fractionation of labeled glycopeptides obtained from Dowex 50 chromatography. The radioactively labeled glycopeptides from Dowex 50 chromatography were pooled and designated as peaks I and II. Peak I was fractionated on DE-52 previously equilibrated with 1 m*M* pyridine (pH 5). The sample was eluted with a gradient (150 ml each) of 1 m*M* pyridine (pH 5) to 0.5 *M* pyridine (pH 5). An aliquot of each fraction was counted for radioactivity.

Fractionation of total lipids. Cellular lipids were purified by the procedure of Ledeen *et al.* (9), as modified by Blumenfeld *et al.* (10). The total lipid fraction (lower phase in C:M:W 3:2:1 extractions) was loaded onto a 0.2 g column of DEAE Sephadex A-25 previously equilibrated with M:C:W (30:15:4). The column was washed with 20 ml of the same solvent; this was pooled with the fraction that did not bind to the DEAE column to obtain the neutral lipid fraction, designated F_1. Another fraction, F_2, containing acidic lipids was eluted with 20 ml M:C:0.8 *M* NH_4OAc in H_2O (30:15:4). For further fractionation of F_1, the sample was dried completely, dissolved in anhydrous chloroform, and applied to a 0.3 g Unisil column equilibrated with chloroform. The column was washed with 30 ml each of chloroform, C:M (95:5), and C:M (1:1) to obtain three fractions.

Results. Liver slices from newborn mice homozygous for the deletion (c^{3H}/c^{3H}) and

from their normal littermates (c^{ch}/c^{ch} or c^{ch}/c^{3H}) were incubated at 37°C for 2 hr with [6-^{3}H]glucosamine. The labeled glycoproteins were extracted, Pronase digested, and the glycopeptides fractioned by Bio-Gel P-6 chromatography as described under Materials and Methods. Figure 1 illustrates the dramatic reduction of labeled glycopeptides observed in the livers of deletion homozygotes (c^{3H}/c^{3H}). The data are expressed in counts per minute of [6-^{3}H]glucosamine incorporated into glycopeptides per milligram liver as a function of elution volume. There are three major glycopeptide peaks which are eluted at 52 ml (1), 70 ml (2), and 81 ml (3) of pyridine formate. A fourth peak is observed at 110 ml and represents unincorporated [6-^{3}H]glucosamine and other labeled amino acids. Similar results were obtained in six separate experiments, both with and without complete medium. In addition, livers from c^{14CoS} and c^{112K} deletion homozygous newborn mice reveal the same reduction in glycopeptide synthesis (data not shown). The three glycopeptide peaks (1, 2, and 3) were pooled and an aliquot counted from each peak to determine total radioactivity contained in the peak. Biosynthesis of glycopeptides in deletion homozygotes (c^{3H}/c^{3H}) amounted to 32, 15, and 37% of normal, peak 1, 2, and 3, respectively (Table I). The biosynthesis of dolichylsaccharides (C:M:W 10:10:3) in livers from c^{3H} deletion homozygotes was 49% of that of their normal newborn littermates (Table I).

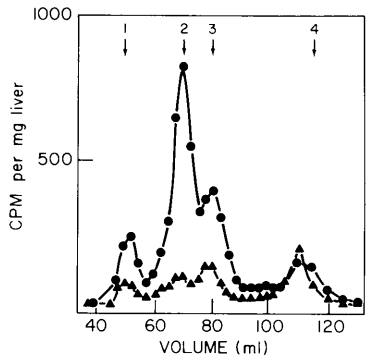

FIG. 1. Bio-Gel P-6 profile of radioactively labeled glycopeptides extracted from the livers of homozygous deletion mutant (▲) c^{3H}/c^{3H}, and normal (●) c^{ch}/c^{ch} littermate mice.

TABLE I. BIOSYNTHESIS OF DOLICHYLSACCHARIDES AND GLYCOPEPTIDES IN NORMAL AND MUTANT NEWBORN MOUSE LIVER

Experimental animal	Dolichylsaccharide	Glycopeptides 1	2	3
	cpm/mg liver/2 hr			
Normal (c^{ch}/c^{ch})	144 (100)	721 (100)	2895 (100)	1372 (100)
Mutant (c^{3H}/c^{3H})	71 (49)	229 (32)	448 (15)	510 (37)

Note. Liver slices were incubated for 2 hr at 37°C in the presence of 40 μCi [6-^{3}H]glucosamine in 1 ml Hanks' BSS under a 95% O_2, 5% CO_2 atmosphere. Values in parentheses are relative to control.

Since no overall difference was observed in incorporation of [6-^{3}H]glucosamine into total lipids between livers of homozygous deletion mutants and livers of their normal littermates (Table II), separate lipid fractions were tested for incorporation differences. Neutral lipid and phospholipid biosyntheses were examined by first pooling the total lipids (lower phases from C:M:W 3:2:1 extractions) of three homozygous normal (c^{ch}/c^{ch}) and two deletion homozygous (c^{3H}/c^{3H}) newborn mice. These lower phase pools were then analyzed for percentage distribution of phosphatidylserine, phosphatidylinositol, cerebrosides, neutral lipids, and phospholipids according to the procedures described under Materials and Methods. The percentage distribution of each of these lipid fractions is very nearly the same in the deletion homozygotes and their normal littermates (Table II). This indicates the absence of gross abnormalities in the biosynthesis of lipids in deletion homozygotes.

In order to determine whether the reduction of incorporation of [6-^{3}H]glucosamine into glycopeptides in the livers of deletion homozygotes is a result of a defect in the biosynthesis or processing of glycopeptides, pulse–chase experiments were performed using liver slices from deletion homozygous newborn (c^{3H}/c^{3H}) and their normal littermates (c^{ch}/c^{ch} and c^{ch}/c^{3H}). Liver slices were pulsed with 40 μCi [6-^{3}H]glucosamine in 1 ml Hanks' BSS for 1 hr at 37°C and chased with 216 μg of nonradioactive glucosamine for 1 hr at 37°C. After lipid extraction and Pronase digestion of the proteinaceous pellet, the glycopeptides ex-

TABLE II. BIOSYNTHESIS OF LIPIDS FROM [6-^{3}H]GLUCOSAMINE IN LIVERS FROM NORMAL AND MUTANT NEWBORN MICE

Experimental animal	Total lipids cpm/animal	Percentage distribution			
		P'serine and P'inositol	Neutral lipids	Cerebrosides	Phospholipids
c^{ch}/c^{ch} (normal) (3)	24,147	6.2	21.7	25.9	16.8
c^{3H}/c^{3H} (mutant) (2)	24,820	4.7	24.4	24.5	20.3

Note. Values in parentheses indicate the number of experimental animals.

tracted from homozygous deletion mutant and normal littermate mice were fractionated on a Bio-Gel P-6 column, as before. A reduction in cpm/mg liver was again observed in the homozygous deletion mutants in each of the three glycopeptide peaks relative to normal littermates (Table III). Peak 2, which is composed of more mature or processed glycopeptides, is reduced by 87% in homozygous mutant mice relative to normal newborn littermates (Table III). These data suggest that the decreased incorporation of [6-^{3}H]glucosamine into glycopeptides in deletion mutant mouse livers is due to a reduction in biosynthesis rather than a failure of processing. Biosynthesis of total lipids and glycoproteins appears to be the same in homozygous (c^{ch}/c^{ch}) and heterozygous (c^{ch}/c^{3H}) normal mice (Table III).

TABLE III. BIOSYNTHESIS AND PROCESSING OF GLYCOPEPTIDES IN LIVERS OF NORMAL AND MUTANT NEWBORN MICE

Experimental animal	Total lipids	Glycopeptides		
		1	2	3
	cpm/mg liver			
Normal: c^{ch}/c^{ch} [1]	304 (100)	92 (100)	590 (100)	791 (100)
c^{ch}/c^{3H} [2]	296 (97)	115 (125)	629 (107)	654 (83)
Mutant: c^{3H}/c^{3H} [1]	220 (73)	40 (43)	75 (13)	132 (17)

Note. Liver slices were pulsed for 1 hr at 37°C with 40 μCi [6-^{3}H]glucosamine in 1 ml Hanks' BSS and chased for 1 hr with 216 μg unlabeled glucosamine. Total lipids and glycopeptides were extracted as described under Materials and Methods. Values in parentheses are relative to normal; values in brackets indicate the number of experimental animals.

The glycopeptides represented by peak 3, obtained by Bio-Gel P-6 chromatography, were analyzed on Dowex 50, eluting with a linear gradient of 1 m*M* to 0.3 *M* pyridine (pH 3.0). This additional fractionation of glycopeptides indicated that deletion homozygous livers again displayed a quantitative reduction of glycoprotein biosynthesis to 20% of normal and no qualitative change (Figs. 2A, D). There are three glycopeptide peaks which do not bind to Dowex at fractions 4, 8–9, and 13. A major peak is eluted at 0.08 *M* pyridine for mutant and normal samples. Glycopeptides contained in peak 2 of the Bio-Gel P-6 fractions were also analyzed on Dowex 50; the results are shown in Figs. 2B and E. A major glycopeptide peak is located at fraction 4 (10 ml of 1 m*M* pyridine) in both mutant and normal samples; however, the mutant peak amounts to 17% of the control. Since this peak contains glycopeptides which did not bind to Dowex 50, it was analyzed on DE-52. Figures 2C and F illustrate the elution profile obtained with a linear gradient of 1 m*M* to 0.5 *M* pyridine. The major glycopeptides of mutant and normal samples are eluted at the same pyridine concentrations; each glycopeptide peak is reduced in the deletion homozygotes. The results of each of these fractionations (Bio-Gel P-6, Dowex 50, DE-52) have allowed us to obtain a "fingerprint" analysis of the glycopeptides synthesized in the livers of homozygous deletion mutants and their normal littermates. The results also indicate that the biosynthesis of glycopeptides is significantly reduced in the mutants, though not totally absent.

Glycoprotein synthesis, requisite for every cell type, is coordinately controlled by polysomes bound to the rough endoplasmic reticulum and the Golgi, both of which are ultra-

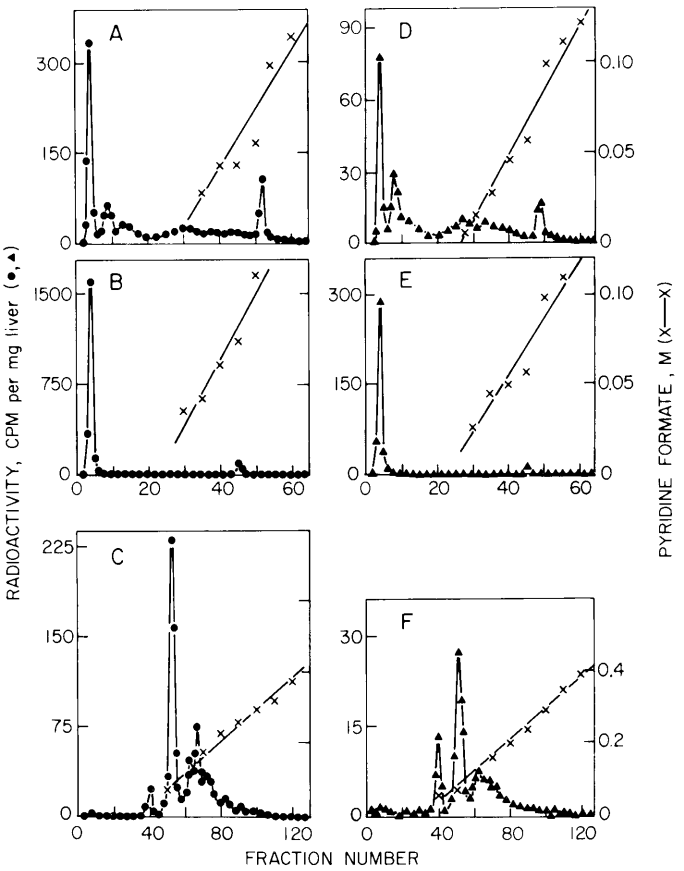

FIG. 2. (A, D) Dowex-50 profiles of radioactively labeled liver glycopeptide peak 3 from Bio-Gel P-6 separation of normal (A) and homozygous deletion mutant (D) littermate mice. (B, E) Dowex-50 profiles of radioactively labeled liver glycopeptide peak 2 from Bio-Gel P-6 separation of normal (B) and homozygous deletion mutant (E) littermate mice. (C, F) DE-52 profiles of radioactively labeled liver glycopeptide peak I from Dowex-50 separation of normal (C) and homozygous deletion mutant (F) littermate mice.

structurally abnormal in liver cells of deletion homozygotes. Electron microscopic studies of other organs have demonstrated them to be ultrastructurally normal (2). Therefore, we investigated glycosylation in another organ to determine if the decreased biosynthesis of glycoproteins observed in mutant mouse liver cells was a general phenomenon. The liver and brain were removed from littermate normal (c^{ch}/c^{ch}) and mutant (c^{3H}/c^{3H}) newborn mice, sliced and incubated for 2 hr at 37°C with 40 μCi [6-^{3}H]glucosamine in 1 ml Dulbecco's medium. Total lipids and glycopeptides were extracted as described under Materials and Methods. Table IV demonstrates the results of these experiments. Glycopeptide biosynthesis is reduced in the livers of deletion mutants to approximately 28% of normal. In contrast, the biosynthesis of glycoproteins in the brain of deletion homozygotes appears to be the same as in homozygous normal littermates.

These data indicate that glycopeptide biosynthesis in the livers of newborn deletion homozygotes is quantitatively reduced by approximately 80% compared to that of their normal littermates. The individual steps in the glycosylation pathway appear to be qualitatively normal, and there is reduction, but not absence, of biosynthesis. Furthermore, the decreased incorporation of [6-^{3}H]glucosamine into glycoproteins is correlated with biosyn-

TABLE IV. BIOSYNTHESIS OF GLYCOPEPTIDES IN LIVER AND BRAIN OF NORMAL AND MUTANT NEWBORN MICE

		Glycopeptide peak III	
Tissue	Animal	cpm/mg/2 hr	Relative percentage
Liver	Normal	1367	100
	Mutant	379	28
Brain	Normal	83	100
	Mutant	101	122

Note. Liver and brain from normal (c^{ch}/c^{ch}) and mutant (c^{3H}/c^{3H}) newborn mice were removed and incubated for 2 hr at 37°C with 40 μCi [6-^{3}H]glucosamine in Dulbecco's medium. Total lipids and glycopeptides were extracted as described under Materials and Methods.

thesis rather than processing. In addition, the reduction in glycoprotein biosynthesis in the deletion homozygotes is liver specific, since the brain does not display the same reduction.

Discussion. The results reported here demonstrate that a major pathway of glycosylation of proteins, requisite for all cell types, is defective in the livers of newborn mice homozygous for a lethal deletion in chromosome 7. The abnormality resides in the biosynthesis of dolichylsaccharides and total glycoproteins. Normally biosynthesis of glycoproteins is strictly controlled in discrete membrane compartments of the cell by a group of linked enzymatic reactions [for review, see (11, 12)]. Polypeptides are synthesized on ribosomes bound to the outer surface of the rough endoplasmic reticulum (RER) and are transported into the lumen of the RER, where the same oligosaccharide is added to every nascent polypeptide chain. One of these initial steps in the dolichol-mediated glycosylation pathway is apparently defective in the livers of the deletion homozygotes, since the synthesis of dolichol oligosaccharides is reduced by approximately 50%. It is tempting to speculate that this could be due to some specific defect in the ultrastructurally abnormal RER membrane system in hepatocytes of deletion homozygotes. For example, such a defect in the hepatocyte RER membrane system could result either in the failure of the ribosome-nascent polypeptide chain signal recognition particle (SRP) to interact with a "docking" protein in the hepatocyte RER, or in a reduction of amount or of biological activity of a specific docking protein.

Processing of glycoproteins begins while they are still in the RER. The Golgi apparatus processes, sorts, and directs glycoproteins to their final destinations in the cell. The data presented in this paper indicate that the hepatocytes of deletion homozygotes, with ultrastructurally abnormal Golgi apparatuses, are capable of processing glycoproteins, even though at a low level. In addition, the pulse–chase experiments suggest that the actual reduction occurs at a step prior to processing. It is possible that transport of the dolichol oligosaccharide precursor (α-mannose$_5$-*N*-acetylglucosamine$_2$-*P*-*P* dolichol) into the lumen of the mutant hepatocyte RER is defective. It seems unlikely that the reduction of glycosylation is due to a lack of one of the multiplicity of enzymes required for normal synthesis of glycoproteins in the dolichol-dependent glycosylation pathway, since glycoprotein biosynthesis in an ultrastructurally normal tissue, i.e., the brain, was found not to be reduced. The observed deficiency, therefore, does not seem to be the result of the absence or mutation of particular structural genes involved in processing glycopeptides, i.e., transferases, mannosidases, etc., but it rather suggests a possible abnormality of a hepatocyte-specific regulatory effect.

Glycosylation defects are restricted to the livers of newborn mice homozygous for the deletion, and hepatocytes are the only cell types that are ultrastructurally abnormal. However, the glycosylation defects cannot be ascribed to a total malfunction of the rough endoplasmic reticulum and/or Golgi apparatus of these mutant cells, since another major biosynthetic pathway involving membranes is normal, that of lipid biosynthesis. Furthermore, earlier experiments demonstrated that total protein synthesis in livers of deletion homozygotes was reduced only slightly compared to that of their normal littermates (3). The glycosylation defects reported here reinforce the cell type specificity of the effects of the homozygous deletions. They may in turn be secondary results of the ultrastructural abnormalities of the subcellular hepatocyte mem-

branes. Alternatively, they may indicate that the DNA sequences included in the deletions are required for the regulation of specific membrane glycosylation processes that function in normal hepatocytes. Such regulatory functions were assigned to these sequences in studies of several liver enzyme deficiencies caused by the same chromosomal deletions (13).

The authors express warmest thanks to Dr. Salome Gluecksohn-Waelsch, without whom this work would not have been possible. Appreciation is also extended to Dr. Olga Blumenfeld for support, Dr. Olga Greengard and Dr. George Acs for reading the manuscript, and to Craig Robinson for providing the mutant material. The work was supported, in part, by grants from the National Institutes of Health (GM-27250 and GM-19100) and the American Cancer Society (CD-38) to S.G.W. and (IN-28W) to P.A.S., and by Grants GM-116389 and AG-00374 to O.B. from the National Institutes of Health.

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Received May 1, 1986. P.S.E.B.M. 1986, Vol. 183.
Accepted June 11, 1986.