Hemagglutination in serum from rectally inseminated animals was significantly lower than that in serum from controls ($P < 0.01$), and IgM, IgG, and IgA titers were similarly diminished. Table 3 shows the outcome of a 5-day intravenous KLH immunization. A reduction in each antibody isotype was associated with animals receiving rectal semen. The differences in IgG, IgA, and IgM binding were all significant at $P < 0.01$, $P < 0.02$, and $P < 0.01$, respectively.

In conclusion, rectal insemination of homologous semen into rabbits had several consequences. Sperm in the rectum evoked the formation of antibodies to sperm despite the presence of seminal plasma, which has been held to be immunosuppressive (*5*). Furthermore, as judged by representative serial sections of tissue taken at autopsy, this response occurred in the absence of insemination-associated trauma to the gut lining or perianal area, possible elements in the etiology of the sperm antibody response seen in homosexual men (*1, 2*). While the rectally inseminated rabbits did not develop the opportunistic infections typically seen in AIDS patients, their reduced responsiveness to T cell–dependent antigens suggests a possible link in this respect. The basis of this effect is not yet known. We have found no significant difference in the rate of clearance of ^{51}Cr-labeled SRBC's in antigen-naïve inseminated and control rabbits, indicating that macrophage interaction with the antigen was not impaired after rectal insemination. Moreover, it is unlikely that the reduced humoral response was due to net lymphocyte depletion, since total PBL counts did not change. However, the appearance of CIC's and antibodies to asialo GM1 in the serum of rectally inseminated rabbits and the fact that serum from the rectally inseminated rabbits inhibited mitogen-induced proliferation of control PBL's in vitro (*13*) raises two possibilities. The first is that CIC's can interfere with immune function (*14*). They have been associated with a number of pathological states (*14*), including AIDS (*1, 2*). Second, the presence of IgG reactive with asialo GM1 in the serum of rectally inseminated rabbits may also be a factor in the decreased immune response. When presented in association with sperm-surface allogeneic histocompatibility antigens, this surface antigen may evoke an autoimmune response leading to the appearance of antibodies reactive with subsets of the T lymphocyte or natural killer cell populations. CIC's or cross-reactive antibodies could foster a degree of suppression by interfering directly with lymphocyte functions or by inducing alterations in the production of soluble mediators of the immune response.

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Location and Chemical Synthesis of a Pre-S Gene Coded Immunodominant Epitope of Hepatitis B Virus

Abstract. *Immunodominant, disulfide-bond independent epitopes recognized by human antibodies to hepatitis B virus (HBV) are located within the 55-residue amino terminal portion (coded for by the pre-S region of HBV DNA) of minor HBV envelope components larger than the major protein constituents encoded by the S gene. A peptide having the sequence of the first 26 amino acids from the amino terminal methionine was synthesized and elicited antibodies (at dilutions of ≥ 1 to 10^5) to the HBV envelope. These antibodies can be utilized for diagnostic tests. The immunogenicity of the peptide was substantially increased by covalent attachment to liposomes. The disulfide bond–independent determinants on sequences coded for by the pre-S gene may be more easily mimicked by peptide analogs than "conformational" determinants on the S-gene product.*

Hepatitis B virus (HBV) infections represent a major public health problem throughout the world. Available vaccines (*1*) produced from the serums of HBV carriers, because of limited resources and production costs involved, do not provide the appropriate means to control and eradicate the disease worldwide. Eventually this may be accomplished by vaccines based on either recombinant DNA technology or on synthetic peptides (*2–6*).

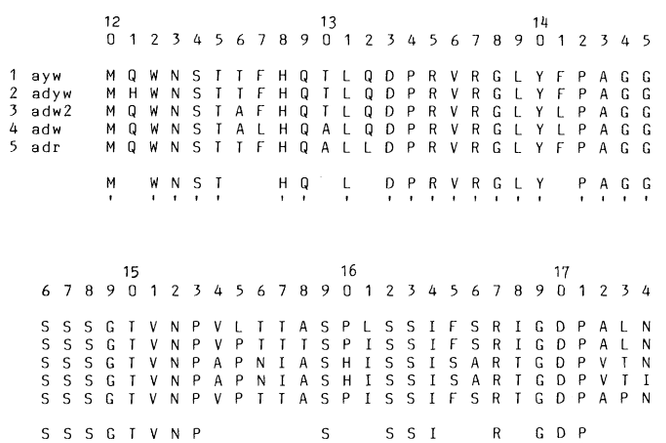
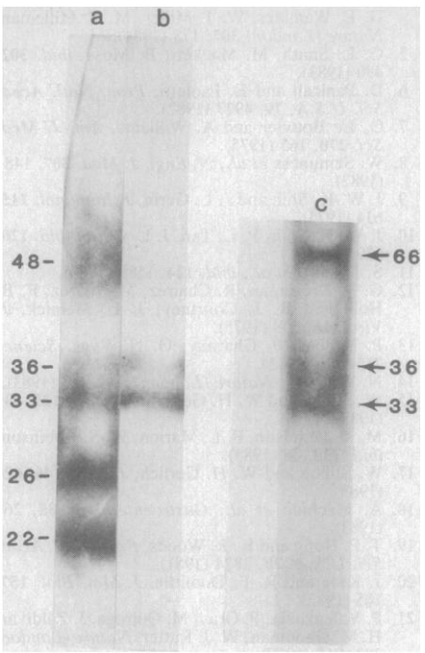


Fig. 1. Amino acid sequences of the translational products of the pre-S gene region downstream from Met 120 as deduced from sequences of HBV DNA (*21, 39–41*). The sequences are presented in one-letter-code words (*42*). Sequences for five distinct HBV subtypes are presented. Line 6 (bottom) shows amino acid residues common to all five subtypes. Single-letter abbreviations for the amino acid residues are A, alanine; R, arginine, N, asparagine; D, aspartic acid; C, cysteine; Q, glutamine; E, glutamic acid; G, glycine; H, histidine; I, isoleucine; L, leucine; K, lysine; F, phenylalanine; M, methionine; P, proline; S, serine; T, threonine; W, tryptophan; Y, tyrosine; V, valine.



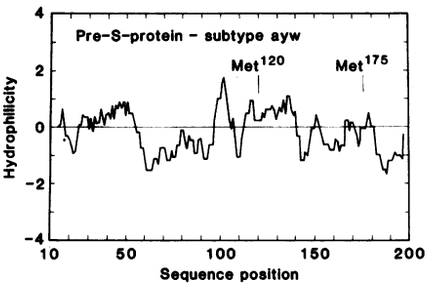
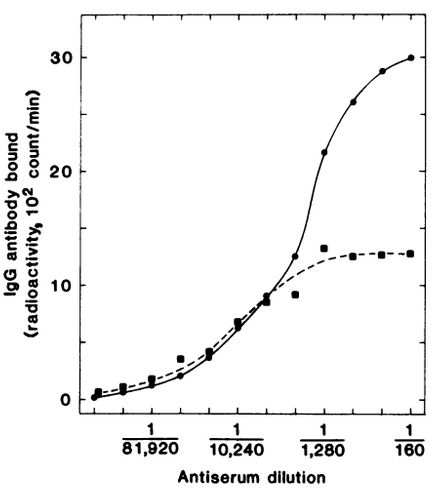


Fig. 2 (left). Polyacrylamide gel electrophoresis of HbsAg. Separated HBsAg polypeptides were (a) stained with silver in situ (*43*), (b) transferred to nitrocellulose and reacted with ^{125}I-labeled antibodies to intact HBsAg, or (c) with antibodies to the synthetic peptide (Pre-S 120–145) followed by ^{125}I-labeled protein A (PA) and submitted to autoradiography. About 20 and 200 μg, respectively, of HBsAg [purified as described (*28*)] were subjected to electrophoresis for silver staining and transfer to nitrocellulose. Before electrophoresis, HBsAg was treated for 30 minutes at 37°C with 2-mercaptoethanol and sodium dodecyl sulfate (10 mg/ml each in 8*M* urea, 0.0625*M* tris, *p*H 7.2). Similar results were obtained with HBsAg alkylated with iodoacetate after reduction. Polypeptides separated by PAGE (*44*) were transferred to nitrocellulose with the use of the TE 42 Transphor unit (Hoefer Scientific Instruments). The transferred proteins were reacted with ^{125}I-labeled human anti-HBs (Abbott Laboratories) as described (*45*), or with unlabeled antibody to the Pre-S(120–145) diluted 1 to 80 in TS containing bovine serum albumin (10 mg/ml) and gelatin (2.5 mg/ml) (TS-PG) for 5 hours at 20°C; to detect bound immunoglobulin G, the nitrocellulose sheet was washed and exposed to ^{125}I-labeled PA (0.4 μCi per 100 ml of TS-PG) for 5 hours at 20°C. In (c), the top arrow indicates another protein (66 kD) reacting with antibody to pre-S(120–145), probably corresponding to a dimer of P33. Polyacrylamide gel electrophoresis was run for a different length of time as compared with (a) and (b). Fig. 3 (right). Profile of relative hydrophilicity corresponding to the amino acid sequence of the pre-S gene product. Profiles for subtypes other than ayw are similar. The portion of the profile after Met^{175} represents the S-gene product.

Several antigenic subtypes of HBV and of subviral (~ 22 nm in diameter) particles (hepatitis B surface antigen) (HBsAg) have been recognized (*7*). All share common (group-specific) envelope epitopes, and the immune response to these appears sufficient for protection against infection by any of the virus subtypes (*8*). The epitope (or epitopes) essential for eliciting virus-neutralizing antibodies has not yet been unambiguously defined. The group specificity is represented by a complex of determinants located on each of the two major ~ 22- and ~ 26-kD constituent proteins (P22 and P26) of the virus envelope and of HBsAg (*9–12*). These proteins have identical amino acid sequences coded for by the S gene of HBV DNA (*13*), except that the larger protein also carries carbohydrate chains. Peptides corresponding to selected segments of the S-gene product were synthesized and elicited antibodies to HBsAg (anti-HBs). However, immunization of chimpanzees with these peptides resulted in only partial protection against HBV infection (*14*). The minor ~ 33- and ~ 36-kD glycoprotein components of HBsAg (P33 and P36) contain the sequence of P22 and have 55 additional amino acid residues at the NH_2 terminal (Fig. 1). This additional sequence is coded by the pre-S region of viral DNA (*15–18*).

We now report (i) that this additional sequence contains a dominant epitope recognized by human antibodies elicited by infection with HBV, and (ii) that a synthetic peptide corresponding to the NH_2-terminal portion of this sequence inhibits the reaction between human anti-HBs and P33 (or P36), is highly immunogenic, and elicits high levels of group-specific antibodies against HBsAg and HBV. The immunogenicity of the peptide is enhanced by covalent linking to liposomes.

Reduced HBsAg, dissociated into its constituent polypeptides, was subjected to polyacrylamide gel electrophoresis (PAGE). As expected (*15–18*), two major and several minor polypeptides were detected by silver staining (Fig. 2a). The separated polypeptides were electrophoretically transferred to nitrocellulose and probed with ^{125}I-labeled antibody to in-



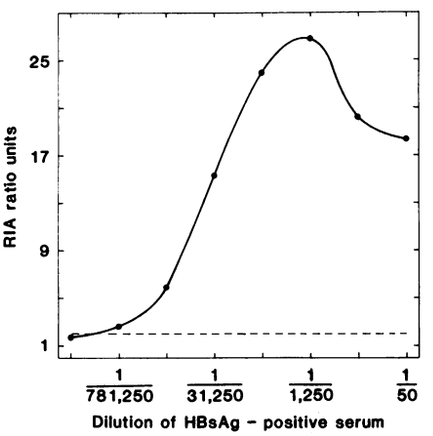


Fig. 4 (left). Radioimmunoassays with serial dilutions of a serum collected 8 weeks after immunization from one of the rabbits immunized with pre-S(120–145) linked to RAT-containing liposomes. Anti-HBs, ■; antibody to pre-S(120–145), ●. Counts per minute corresponding to distinct dilutions of the preimmune serum were subtracted from the number of counts per minute corresponding to dilutions of antibody to pre-S(120–145); the difference was plotted. The end-point titer of the serum (1/163, 840) corresponds to its highest dilution at which the number of counts per minute were more than 2.1 times higher than those corresponding to the same dilution of the preimmune serum. Similar results were obtained with serums from two other rabbits immunized in the same way. Antibody titers of serums from rabbits injected with free peptide or with liposomes not having RAT groups were about ten times lower. Fig. 5 (right). Diagnostic test based on polystyrene beads coated with antiserum to pre-S(120–145). Serial dilutions of an HBsAg-positive serum in a mixture of normal human and rabbit serum each diluted 1 to 10 in TS were tested. ^{125}I-Labeled human anti-HBs (Abbott) was used in the test, performed essentially as described for the AUSRIA II diagnostic kit (Abbott). Results are expressed as RIA ratio units, determined by dividing the number of counts per minute corresponding to positive samples by the number of counts per minute corresponding to normal serum controls. The end-point titer corresponds to the highest dilution at which the RIA ratio was ≥ 2.1 (broken line). The end-point titer of the serum as determined by the AUSRIA test was ~ 1/10^6. Negative results were obtained with control beads coated with normal rabbit immunoglobulin G.

tact HBsAg (anti-HBs). Surprisingly, the 33 and 36 kD (P33 and P36) rather than the two most abundant polypeptides reacted preferentially with this antibody (Fig. 2b). This suggested the presence of disulfide bond–independent antigenic determinants involving amino acid sequences that are not coded for by the S gene of HBV DNA.

The location of antigenic determinants on proteins may be predicted from computing the relative hydrophilicity along the amino acid sequence (*19, 20*). Results of such computation (*20*) for the translation product of the pre-S region are shown in Fig. 3 and suggest the location of antigenic determinants within residues 120 to 140. The segment corresponding to residues 120 to 145 (Fig. 1) [pre-S(120–145)] was selected for synthesis. The peptide corresponding to subtype *adw* (*21*) containing an additional Cys residue at the COOH-terminal, added for convenience of coupling to carriers, was synthesized by an improved solid-phase technique (*22–24*). Quantitative Edman degradation (*25*) of the assembled peptide-resin revealed a high efficiency of chain assembly (*26*) which proceeded at a > 99.7 percent efficiency at each step. High-performance liquid chromatography (HPLC) of the peptide cleaved off the resin revealed a single major peak corresponding to > 85 percent of peptide material absorbing light at 225 nm.

Immunization of rabbits with either free or carrier-bound pre-S(120–145) resulted in an antibody response in all animals against both the peptide and HBsAg (*27–29*). The antibody titer increased approximately fourfold every 2 weeks from week 2 to week 8 after primary immunization. These antibodies were still detectable when some of the final antiserums (obtained 8 weeks after primary immunization) were diluted up to 1.6×10^5-fold (Fig. 4). Pre-S(120–145) or antiserum to pre-S(120–145) inhibited the reaction between ^{125}I-labeled anti-HBs and P33 (or P36) as indicated by the complete or partial inhibition of binding of ^{125}I-labeled anti-HBs to P33 and P36 separated by PAGE (data not shown). Antiserum to pre-S(120–145) agglutinated HBV particles (concentrated from serum by ultracentrifugation) as indicated by electron microscopy. The immune complexes contained HBV DNA detected by blot dot hybridization (*30*) (data not shown). The antibody to pre-S(120–145) also served as a specific probe for the detection of P33 and P36 (Fig. 2c). It was also utilized for the development of a diagnostic test for the direct detection of antigens coded for by the pre-S gene in serums of HBV-infected individuals (Fig. 5). Similar results were obtained with serums containing HBsAg subtypes *ad* and *ay*, indicating that the synthetic peptide with the sequence corresponding to subtype *ad* (Fig. 1) carried common group-specific antigenic determinants.

Cleavage of disulfide bonds within the envelope proteins of HBV results in (i) a substantial decrease of binding of polyclonal (*31–34*) and of some monoclonal (*35*) antibodies elicited by intact HBsAg and (ii) reduction of immunogenicity (*36*). However, some epitopes are resistant to reduction of disulfide bonds (*37*) and are common to all antigenic subtypes of HBV, but their location on envelope components of HBV has not been determined. Our results provide evidence that antigenic determinants independent of disulfide bonds are present on the amino terminal portion (coded for by the pre-S gene of HBV DNA) of the minor HBsAg proteins P33 and P36.

These disulfide bond–independent determinants represent the dominant epitopes on reduced and dissociated HBsAg that reacts with human anti-HBs. They are mimicked with high fidelity by pre-S(120–145), which elicits antibodies to HBsAg about 400 times more efficiently than a previously synthesized peptide analog of a segment of the S-gene product (both antibodies were measured by identical methods) (*29*). No precedent exists for such high levels of virus-recognizing antibodies to a synthetic peptide analog of an HBV protein.

The pre-S gene is the most divergent among all regions of hepadnavirus genomes (*38*). Therefore, the product of this gene may play a role in the species specificity of HBV and possibly may be involved in attachment of HBV to susceptible cells. If so, synthetic peptide analogs corresponding to selected segments of the translational product of the pre-S gene are promising immunogenes for eliciting protection against HBV infection.

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27. For immunoassays and linking to carriers, the peptide was treated with 2-mercaptoethanol and separated from low molecular weight components by chromatography on Sephadex G-10 (*28*). Groups of two to three rabbits were immunized with either free pre-S(120–145) or with the peptide linked to cysteine-activated liposomes (containing stearylamine, dilauroyl lecithin, and cholesterol) that had been fixed with glutaraldehyde, and either did or did not have attached RAT (L-tyrosine azobenzene-*p*-arsenate) groups for enhancing antibody responses to haptens (*29*). The immunization schedule involving biweekly immunizations and the use of complete and Freund's incomplete adjuvant was the same as described (*28*). Antibodies to HBsAg in serums of rabbits immunized with pre-S(120–145) were tested by a double-antibody radioimmunoassay (RIA) with HBsAg-coated polystyrene beads and ^{125}I-labeled antibody to rabbit immunoglobulin G (*28*). Antibodies to the peptide were tested by a similar test, except that 2.5 mg of a cellulose-peptide conjugate was used instead of coated beads. This conjugate was prepared as follows: sulfhydryl cellulose (0.5 g) was suspended in 0.1*M* sodium acetate, *p*H 5, and mixed with 0.25*M* *N*-*N*′-*p*-phenylenedimaleimide (2.5 ml) in dimethylformamide for 1 hour at 30°C and then washed with 0.1*M* phosphate containing 10 m*M* EDTA, *p*H 7.0. The cellulose derivative was suspended in 10 ml of this phosphate-EDTA buffer containing 5 mg of reduced pre-S(120–145) and mixed for at least 16 hours at 20°C. The cellulose derivative was extensively washed and suspended in a solution of 0.14*M* NaCl, 10 m*M* tris, and 3 m*M* NaN_3 (TS). The final preparation contained 8 mg of pre-S(120–145) per gram of cellulose.
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Potentiation of Opiate Analgesia and Apparent Reversal of Morphine Tolerance by Proglumide

Abstract. *Exogenous cholecystokinin selectively antagonizes opiate analgesia, which suggests that endogenous cholecystokinin may act physiologically as an opiate antagonist and may play a role in opiate tolerance. The use of the selective cholecystokinin antagonist proglumide provided a test of these hypotheses in rats that were either inexperienced with or tolerant to opiates. Proglumide potentiated analgesia produced by morphine and endogenous opiates and seemed to reverse tolerance. These results suggest that endogenous cholecystokinin systems oppose the action of opiates.*

Cholecystokinin (CCK) selectively acts as an opiate antagonist in rats when administered either systemically or centrally (*1*). These findings, plus the finding of CCK in neural areas previously implicated in pain modulation (*2*), led us to hypothesize that endogenous CCK may function physiologically to oppose the analgesic effects of opiates. These data also suggest that tolerance resulting from repetitive opiate administration may be due to a compensatory increase in the activity of CCK systems. We tested these hypotheses by examining the effect of proglumide, a competitive CCK receptor antagonist (*3*), on opiate analgesia produced in rats either naïve to or tolerant of opiates.

We found that endogenous CCK functions physiologically as an opiate antagonist and plays a role in opiate tolerance. The observation that proglumide can potentiate opiate analgesia has implications for the administration of narcotics for both acute and chronic pain.

We initially examined the effect of five doses of proglumide (0.001, 0.01, 0.1, 1, and 5 μg) delivered intrathecally onto the lumbosacral cord (*4*) on analgesia (*5*) produced by intrathecally administered morphine (1 μg in 0.5 μl saline). Proglumide or equivolume vehicle [0.5 μl of 0.4 percent dimethyl sulfoxide (DMSO) and buffer] (*6*) was injected 10 minutes before and again immediately before morphine administration. A biphasic dose-response function was observed. Dose-related potentiation was produced by 0.001- and 0.01-μg doses (Fig. 1A), no effect by 0.1 μg, and dose-related attenuation by 1.0 and 5.0 μg (*7–9*).

Since these results suggest that endogenous CCK was able to oppose the antinociceptive actions of intrathecal morphine, we tested whether endogenous CCK might also attenuate analgesia induced by endogenous opiates. We examined the effect of 0.01 μg of proglumide (two intrathecal injections, 10 minutes apart) on analgesia produced by spinal release of endogenous opiates (front-paw footshock-induced analgesia) (*10*) and on

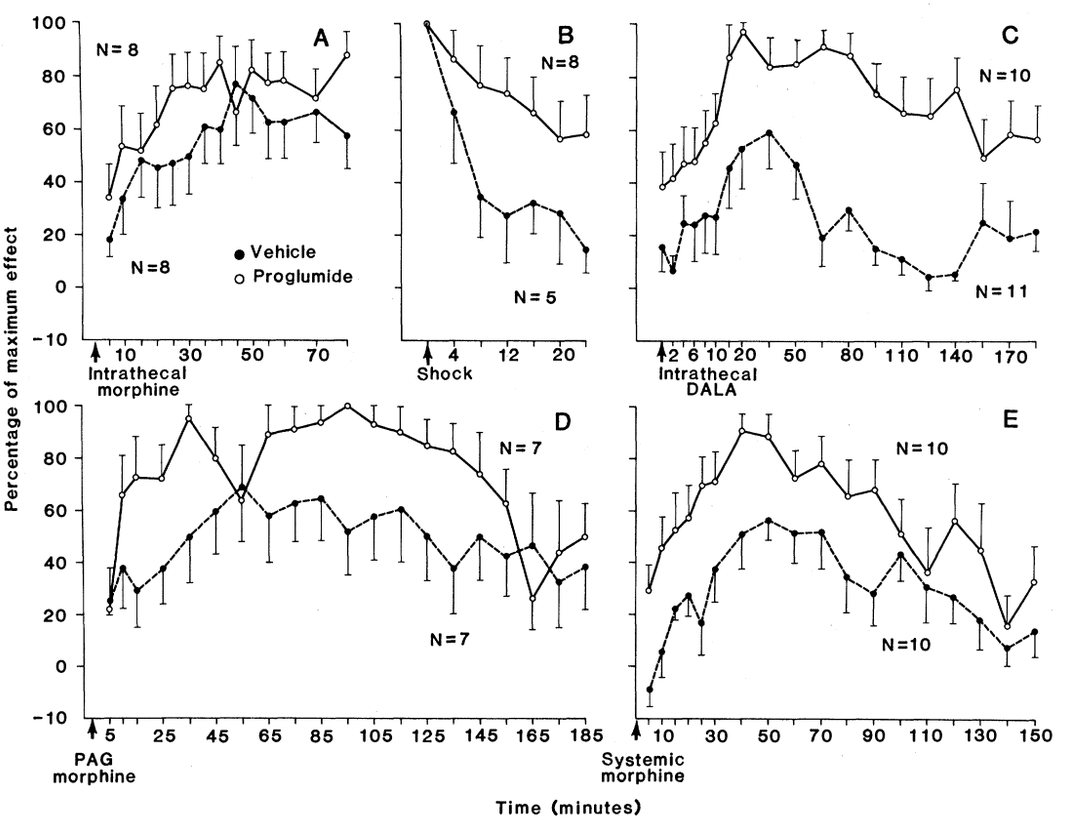

Fig. 1. Potentiation (mean ± standard error) of opiate analgesia by proglumide (two injections of 0.01 μg, the optimum potentiating dose). (A) Enhancement of intrathecal morphine analgesia by intrathecal proglumide. (B) Enhancement of front-paw footshock-induced analgesia by intrathecal proglumide. (C) Enhancement of intrathecal DALA analgesia by intrathecal proglumide. (D) Enhancement of PAG morphine analgesia by PAG proglumide. (E) Enhancement of systemic morphine analgesia by systemic proglumide. Data were evaluated by analyses of variance; for each comparison, $P < 0.0001$.