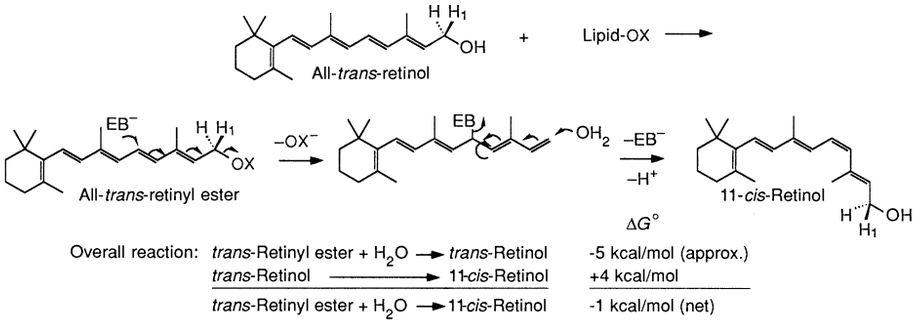

Scheme 2. A coupled reaction could drive the isomerization. Abbreviations: EB^-, enzyme active–site nucleophile; and $\Delta G°$, Gibbs free energy.

(*8*). This result is only understandable if C–O bond cleavage occurs during isomerization.

We next attempted to determine the nature of the putative ester substrate of the isomerase. Previously we had shown that when all-*trans*-retinol was added to pigment epithelium membranes it was rapidly converted into retinyl esters, mainly the palmitate ester, before any isomerization was observed (*3, 4*). However, at least in the amphibian case, a small amount of all-*trans*-retinol and all-*trans*-retinal remained as well (*4*). We reinvestigated the processing of all-*trans*-retinol by bovine membranes with some alterations from previous techniques. The membranes were first irradiated with UV light and washed to remove the endogenous retinoids and nicotinamide cofactors. Under these conditions a true tracer experiment can be performed. The addition of [11,12-^{3}H]all-*trans*-retinol to the membranes led to the virtually complete conversion of this material into all-*trans*-retinyl esters (Fig. 2A). Approximately 3% of the all-*trans*-retinol remained, even though an extraction technique was used that overestimates the amount of retinols with respect to retinyl esters (*3*). The percentage of retinyl esters eventually decreased, with a concomitant increase in 11-*cis*-retinol (Fig. 2A). The percentage of all-*trans*-retinol also increased with time, but this was probably due to back isomerization from its 11-*cis*-congener. When the membranes were treated with [11,12-^{3}H]all-*trans*-retinyl palmitate, processing to 11-*cis*-retinol occurred (Fig. 2B). In separate experiments it was shown that [11,12-^{3}H]all-*trans*-retinyl oleate could be converted into 11-*cis*-retinol but not 11-*cis*-retinyl oleate. This is further evidence for the lack of a direct ester-ester isomerization route. These results suggest that under normal physiological conditions a retinyl ester is directly isomerized to 11-*cis*-retinol. Other experiments are consistent with this view. For example, we have not been able to dissociate retinyl ester synthetase activity from isomerase activity during purification of the detergent solubilized materials (*7*), and different reagents, such as ethanol, *p*-hydroxymercuribenzoate, and hydroxylamine, affect both activities in a parallel fashion (*11*). Finally, structure-activity studies are also consistent with the proposed mechanism (*12*).

Our results show that the biological formation of 11-*cis*-retinol requires the input of metabolic energy and that the energy source for this conversion is membrane-derived. A novel group transfer mechanism has been postulated that uses the metabolic energy of an ester moiety of the phospholipid to drive this process. The proposal that membranes may serve as an energy source reveals a previously unsuspected biological function of membranes.

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A G Protein Gamma Subunit Shares Homology with ras Proteins

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Guanine nucleotide binding proteins (G proteins) that transduce signals from cell surface receptors to effector molecules are made up of three subunits, α, β, and γ. A complementary DNA clone that encodes a 71–amino acid protein was isolated from bovine brain; this protein contains peptide sequences that were derived from the purified γ subunit of G_i and G_o. The primary sequence of this G protein γ subunit (Gγ) has 55 percent homology to the γ subunit of transducin (Tγ) and also has homology to functional domains of mammalian ras proteins. The probe for isolating the clone was generated with the use of the polymerase chain reaction (PCR). The extent of divergence between Tγ and Gγ, the isolation of homologous PCR-generated fragments, and the differences between the predicted amino acid sequence of Gγ and that derived from the γ subunit of G_i and G_o indicate that γ subunits are encoded by a family of genes.

G PROTEINS ARE GENERALLY FOUND associated with cell membranes and are activated by a class of cell surface receptors that includes the β-adrenergic receptor, rhodopsin, and the muscarinic acetylcholine receptor. The activated G protein transduces signals from these receptors to effectors such as phospholipases, guanosine 3′,5′-monophosphate (cGMP) phosphodiesterase, adenylyl cyclase, and ion-conducting channels (*1*). In well-characterized systems such as the G protein activation of cGMP phosphodiesterase and adenylyl cyclase, the G protein α subunit in the activated state binds guanosine 5′-triphosphate, dissociates from βγ, and directly modulates effector function (*2*). On the basis of these

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data, it has been suggested that the α subunit is the active species in G protein function.

The role of the G protein βγ subunit complex has been less clear. There are indications that it might act independently on effectors (*1*). There is also evidence that the βγ complex is required for the interaction of the α subunit with the receptor (*3*). Nine different α subunits have been characterized (*4*). However, only two different β subunits, which show a high degree of homology, have been identified by isolating cDNAs, one coding for a 36-kD protein and another for a 35-kD protein (*5*). The γ subunits have been poorly characterized despite suggestions that there are at least three different types (*6, 7*). The only primary structure of a γ subunit known so far is that of Tγ, the γ subunit associated with transducin (the G protein specific to vertebrate rod outer segments). The Tγ subunit was characterized by isolation of its cDNA from bovine retina (*8*). We report the isolation of a cDNA coding for a G protein γ subunit by a method based on the polymerase chain reaction (PCR) (*9*). A fragment of the coding sequence was amplified by PCR, and this in turn was used as a probe to isolate the cDNA for the entire protein (*10*).

The α, β, and γ subunits of highly purified G_i and G_o proteins from bovine brain (*7, 11, 12*) were separated by SDS–polyacrylamide gel electrophoresis (PAGE). The proteins were transferred to nitrocellulose, and the band corresponding to the γ subunit was cut out (*13*) and then digested with trypsin; the resulting peptides were separated by high-performance liquid chromatography (HPLC). Three of the most prominent peptides were then sequenced (*14, 15*). The amino acid sequences of these peptides were AAADLK (PepA), SEADAKEDPL (PepB), and EDPLLTPVPASENP (PepC) (*16*). A sequence of 20 amino acids (PepD) was derived from the two overlapping sequences, PepB and PepC. The PepD sequence was homologous to a stretch of amino acids in Tγ from Val^{44} to Pro^{63}, and eight amino acids were at identical positions in the two sequences. The PepA sequence could not be aligned with any portion of the Tγ sequence.

To amplify the DNA fragment coding for a part of the amino acid sequence of PepD, we synthesized sense and antisense primers as degenerate oligonucleotides specific to different regions of this sequence and then used PCR on three different templates that were prepared from bovine brain polyadenylated [poly(A)$^+$] RNA and bovine genomic DNA (Fig. 1, A and B). Regions of PepD were chosen so that the primers would have minimal degeneracy and would be separated sufficiently to generate a PCR-amplified product that could be distinguished in length from the primers. On the basis of the amino acid sequence the amplified product was expected to be 56 bases long. All three templates generated a fragment that was approximately of the expected size and also a fragment of ~100 bases (Fig. 1B). In addition, the primers amplified DNA of ~200 bases from genomic DNA alone. The DNA fragments of ~50 to 60 bases were isolated from the three different reactions, pooled, subcloned into a plasmid, and sequenced. The nucleotide sequence between the two primers of one of these fragments, PCR3-3, codes precisely for the amino acid sequence found in PepD (Fig. 1C).

It was expected that the PCR product PCR3-3 could have incorporated primers with mismatched bases in comparison to the corresponding regions of the cDNA (*10*). Because this could have a destabilizing effect if the probe were used to screen a bovine brain cDNA library, a new probe was synthesized by amplifying the PCR3-3 insert from the plasmid with a mixture of the oligonucleotides (a) and (c) as primers (Fig. 1A). This probe hybridized to two clones, λBG5 and λBG6 (*17*), which contained 0.5- and 1.5-kb inserts, respectively, as seen from Eco RI digestion. The nucleotide sequence of the fragment PCR3-3 was present between nucleotide 241 and 296 of the fragment from λBG5 (Fig. 2A). There were six mismatches between PCR3-3 and the Gγ cDNA (Fig. 1C). When this sequence was translated in one particular reading frame, the sequences of PepA and PepD were found to be encoded by the stretch of nucleotides from 214 to 297. There were three differences between the predicted amino acid sequence of Gγ and those of the peptides; Met instead of Lys at position 38, Cys instead of Ser at position 41, and His instead of Asp at position 44 (Fig. 2A). Two independently isolated cDNA clones of different sizes (λBG5 and λBG6) had precisely the same sequences coding for these amino acids.

The peptides from the γ subunit of G_i and G_o were obtained by trypsin digestion. A comparison of the Gγ amino acid sequence with the peptide sequences showed that the amino acid in Gγ immediately upstream of the sequence corresponding to PepA is Lys and the COOH-terminal amino acid of PepA is also a Lys, as would be expected from trypsin digestion. In Gγ, Met is present in place of the COOH-terminal Lys of PepA. The Gγ sequence upstream of the region corresponding to PepB does not have

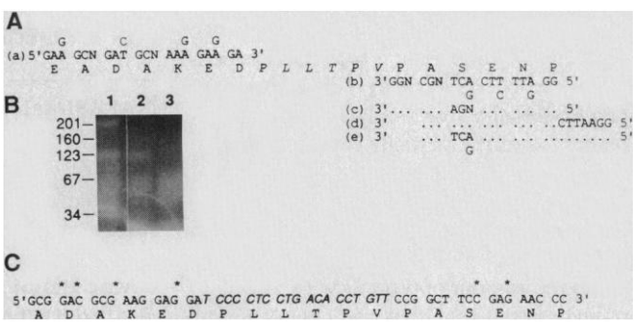


Fig. 1. PCR amplification of a fragment of the cDNA coding for Gγ. (**A**) Primers specific to portions of the amino acid sequence of PepD; (a), (b), and (c) were used for the PCR reaction; (d) and (e) were used for reverse transcription. Dots indicate that the sequence is the same as the one above. Bovine brain poly(A)$^+$ mRNA was reverse-transcribed after first annealing the primer to the mRNA by heating the two together at 90°C for 4 min and then chilling. The reaction mixture (50 μl) contained 50 m*M* tris-HCl (*p*H 8.3), 75 m*M* KCl, 10 m*M* dithiothreitol, 3 m*M* $MgCl_2$, 600 U of Moloney–murine leukemia virus reverse transcriptase (Bethesda Research Laboratories), 500 μ*M* of each deoxynucleotide, 10 μCi of [α-^{32}P]dCTP (deoxycytidine 5′-triphosphate) (~3000 Ci/mmol), 0.3 μg of mRNA, and 200 ng of the mixed oligonucleotides (d) and (e) as primers or 200 ng of oligo(dT) (12 to 16 nucleotides in length). The reaction was performed at 37°C for 105 min, and then 1 μl of reaction mixture was used to estimate the amount of [α-^{32}P]dCTP incorporated and thus the efficiency of reverse transcription. DNA from the reaction mixture was precipitated and then resuspended in 20 μl of water, and 1 μl of this was used as the template for PCR. (**B**) Agarose gel analysis of PCR-amplified DNA fragments from three different templates with specified primers (size is expressed in bases). (Lane 1) Bovine genomic DNA (testis) and primers (a) and (c); (lane 2) bovine brain mRNA reverse-transcribed with (d) and amplified with (a) and (c); (lane 3) bovine brain mRNA reverse-transcribed with oligo(dT) and amplified with (a) and (c) (*22*). In each case a typical 100-μl reaction volume consisted of 2.5 U of Taq polymerase (Perkin-Elmer Cetus), 1× PCR buffer [50 m*M* KCl, 10 m*M* tris-HCl (*p*H 8.3), 1.5 m*M* $MgCl_2$, and 0.1% gelatin], 200 μ*M* of each deoxynucleotide, and 5 to 7 μ*M* primer. Reaction mixes were denatured at 94°C for 5 to 10 min before adding the enzyme and deoxynucleotides. The number of cycles varied between 25 to 30, and each cycle included denaturation for 1 min at 94°C, annealing for 1 min at 46°C, and extension for 30 s at 55°C or 20 s at 72°C. (**C**) Sequence of an amplified fragment. Sequence between PCR primers is in italics. Amplified DNA was electrophoresed on low melting point Nusieve (Seaplaque) agarose gels (4%); appropriate bands were cut out, and the DNA was eluted. The DNA fragments were then ligated to linkers, purified, and cloned into Bluescript (Stratagene). Double-stranded DNA plasmids containing inserts were isolated and sequenced with Sequenase (U.S. Biochemicals). Asterisks indicate mismatches between the amplified fragment and Gγ cDNA.

either Lys or Arg. These differences suggest that a homolog of the Gγ cDNA isolated here codes for a γ subunit that has amino acid sequences corresponding to the peptides PepA and PepB. The deduced primary structure of Gγ has 71 amino acids with a predicted molecular mass of 9100 daltons.

An alignment of the Gγ amino acid sequence with that of Tγ shows that the primary structure of Gγ diverges considerably from that of Tγ (Fig. 2B). The Gγ sequence has 55% homology on the basis of similarity of amino acids and 32% identity. The extent of divergence between the two subunits is similar to that between two of the most structurally and functionally divergent G protein α subunits, those from G_s and G_o (50% similarity and 35% identity) (*18*). The extent of divergence in the primary structures of the two γ subunits might be an indication of differences in their functions.

When the amino acid sequence of Gγ is compared with that of the mammalian K-ras, H-ras, and N-ras proto-oncogene products (*19*), the NH_2-terminal third of the Gγ amino acid sequence is homologous to a stretch of amino acids in the middle (amino acids 83 to 101) of the ras proteins. The homology is 45% by identity when the first 20 amino acids of Gγ are compared with the K-ras and H-ras proteins (Fig. 3). The region between amino acids 77 to 93 of ras is known to be essential for its ability to transform (*20*) and is a putative effector binding region (*19*). Although the first 15 amino acids of Gγ show 33% identity with ras proteins, the same stretch of amino acids shows little homology to the corresponding sequences of Tγ (Fig. 3). There are no identical amino acids in the two γ subunit sequences in this region. Furthermore an exon-intron junction in all three mammalian ras genes, K-*ras*, H-*ras*, and N-*ras*, is located at the point where the two γ subunit sequences begin to diverge (Fig. 3). Distinctive NH_2-terminal amino acids of the γ subunits of G proteins could potentially interact with an effector or with a receptor that could also be associated with ras proteins. The COOH-terminus of the putative Gγ protein has the sequence Cys-a-a-X similar to ras as well as related proteins (a is an aliphatic amino acid and X is any amino acid). This sequence is also present in Tγ. The cysteine in this sequence is known to be acylated in the case of some ras proteins and is essential for membrane association and transforming capability (*19*). This is consistent with the notion that the γ subunits share similar COOH-terminal regions that are required for membrane association and possess different and specific NH_2-terminal sequences. In addition, Gγ possesses a proteolytic cleavage site B-X-B-B (B is a basic amino acid residue) (amino acids 62 to 65) that is present in ras proteins (*21*) (Fig. 2A). The COOH-terminal sequence Cys-a-a-X in Gγ is preceded by two phenylalanines, in contrast to Tγ, which has two glycines at these positions (Fig. 2B). The attachment of a fatty acid to the Cys of Gγ would make this region very hydrophobic in comparison to Tγ. This is consistent with evidence that the βγ complex of G_i and G_o is hydrophobic and that of transducin is hydrophilic.

Hybridization of the cDNA for Gγ to mRNA from different bovine and mouse tissues showed that a 4.4-kb transcript specific to this cDNA is expressed at higher levels in brain compared to some other tissues (Fig. 4). The Tγ transcript is specifi-

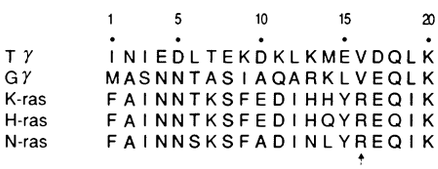


Fig. 3. The NH_2-terminal 20 amino acids of Gγ aligned with homologous stretches from the ras proteins and Tγ. Amino acids of Gγ are numbered. This alignment was obtained by comparing the segment of Gγ with the amino acid sequence of each of the other proteins by using the Gap program (*24, 25*). Conserved residues are shaded (*26*). The arrow indicates the location of the exon-intron junction in the genes for all three ras proteins.

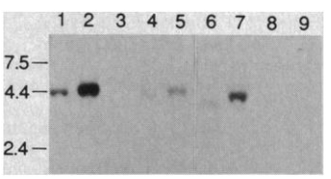
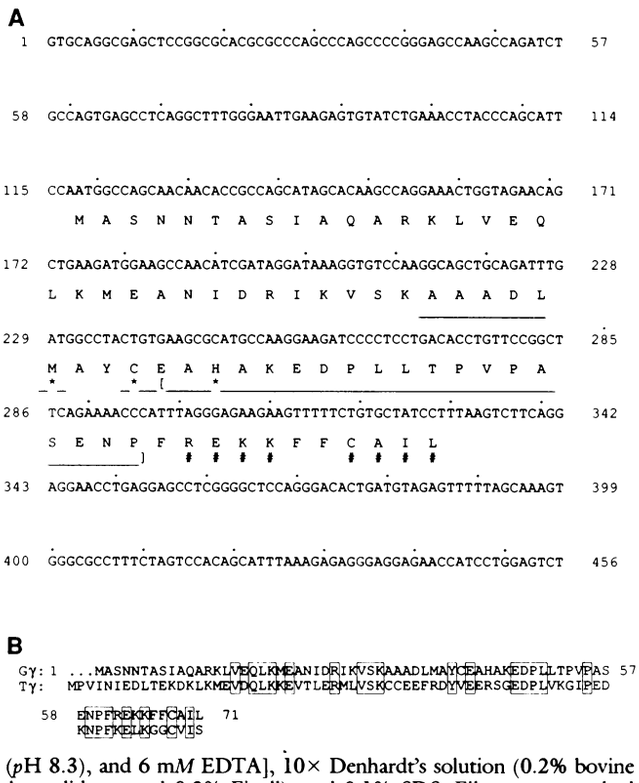


Fig. 4. RNA blot analysis of bovine and mouse tissues with the cDNA coding for Gγ (sizes are expressed in kilobases). (Lanes 1 to 6) Poly(A)$^+$ RNA from the following bovine tissues (micrograms of RNA per lane are in parentheses after tissue): (Lane 1) adrenal (1); (lane 2) brain (1); (lane 3) heart (2); (lane 4) kidney (2); (lane 5) liver (2); and (lane 6) testis (4). (Lanes 7 to 9) Poly(A)$^+$ RNA from mouse tissues: (Lane 7) brain (2); (lane 8) eye (2); and (lane 9) liver (2) (*27*). The RNA samples were electrophoresed in the presence of ethidium bromide (100 μg/ml) through a 1.2% agarose gel containing formaldehyde. The gel was photographed and blotted with a nitrocellulose filter (*23*). The prehybridization at 65°C and hybridization at 42°C were performed in a solution containing 5× SSC, 25 m*M* potassium phosphate (*p*H 7.6), 50% formamide, 10% dextran sulfate, 0.2% SDS, and denatured salmon sperm DNA (100 μg/ml). The filter was washed sequentially at increasing levels of stringency. The final wash was at 65°C with 0.1× SSC and 0.1% SDS for 30 min. The cDNA insert was amplified with primers specific to Bluescript and labeled with ^{32}P by primer extension (*23*).

Fig. 2. (**A**) Nucleotide sequence and deduced amino acid sequence of Gγ from bovine brain. Only a portion of the noncoding regions is shown. The amino acid sequences derived from the peptides are underlined. Asterisks indicate that these amino acids are different in the peptides. Number signs (#) denote domains conserved in mammalian ras proteins. (The NH_2-terminal homology to ras is shown in Fig. 3.) The region amplified by PCR is bracketed below. A bovine brain cDNA library in the phage vector λgt10 was screened with the probe described in the text. The PCR-amplified probe was purified and labeled with [γ-^{32}P]ATP (adenosine triphosphate) and polynucleotide kinase (*23*). Nitrocellulose filters with plaques were prepared (*23*), prehybridized at 65°C, and hybridized at 57°C in a solution containing 6× SET [0.9*M* NaCl, 90 m*M* tris-HCl (*p*H 8.3), and 6 m*M* EDTA], 10× Denhardt's solution (0.2% bovine serum albumin, 0.2% polyvinylpyrrolidone, and 0.2% Ficoll), and 0.1% SDS. Filters were washed three times for 5 min each at room temperature and once at 57°C for 1 min. (**B**) Alignment of the Gγ amino acid sequence with that of Tγ was obtained by using the computer program Gap (*24, 25*). Identical residues are in blocks.



cally expressed in the retina.

The isolation of more members of the γ subunit family will help to clarify the relation among the three G protein subunits. To date, G proteins have been distinguished by their α subunits. If there are many diverse members of the γ subunit family, a particular α subunit might be associated with more than one species of γ, and each of these combinations could have a different function. This combinatorial association would increase the diversity of G protein functions.

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13. Even though the band corresponding to the γ subunit was discrete on SDS-PAGE, it has been shown by others [P. C. Sternweis and J. D. Robishaw, *J. Biol. Chem.* **259**, 13806 (1984)] that the γ subunit of the purified bovine brain G protein is heterogeneous.
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15. The βγ complex fractionated from the G proteins G_i and G_o copurified from bovine brain (*7, 11*) and was used as the source of the γ subunit. The γ subunit was isolated by electrophoresing 25 μg of a mixture of G_i and G_o on a 10 to 20% SDS-polyacrylamide gel. The proteins were transferred to nitrocellulose, stained with Ponceau S, and the band corresponding to γ was cut out. This band was digested directly in a solution containing trypsin. The resultant peptides were separated by HPLC on a C_4 hydrophobic column. Peptides corresponding to 3 peaks out of 14 were sequenced on a gas-phase sequenator. Initial attempts to sequence the protein from the NH_2-terminus showed that the terminus was blocked, necessitating the sequencing of internal peptides.
16. Abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
17. Analysis by PCR of phage from these plaques with primers specific to the fragment PCR3-3 and λ vector sequences gave an estimate of the length of the sequence 5′ to the PCR3-3 sequence.
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27. The same probe hybridized to a 4.4- and a 3-kb RNA from bovine lung and spleen.

28. We thank J. K. Northup for the G_i and G_o proteins, R. Miake-Lye for the cDNA library and RNAs, B. W. Birren for the genomic DNA, M. Strathmann for discussions, and T. Amatruda and T. Wilkie for comments on the manuscript.

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Oxygen Radicals in Influenza-Induced Pathogenesis and Treatment with Pyran Polymer-Conjugated SOD

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The pathogenicity of influenza virus infection in the mice involves, at least in part, overreaction of the immune responses of the host rather than a direct effect of virus multiplication. Xanthine oxidase, which is responsible for the generation of oxygen free radicals, was elevated in serum and lung tissue of mice infected with influenza virus. To test the theory that oxygen-free radicals are involved in pathogenesis, free radicals were removed by injecting superoxide dismutase (SOD), a specific superoxide radical scavenger, which was conjugated with a pyran copolymer. The conjugate protected mice against a potentially lethal influenza virus infection if administered 5 to 8 days after infection. These findings indicate that oxygen radicals are important in the pathogenesis of influenza virus infection, and that a polymer-conjugated SOD has therapeutic potential for this virus infection and other diseases associated with free radicals.

A TYPICAL CHARACTERISTIC OF INfluenza virus infection in the mouse lung is the presence of areas of surface consolidation, which under the microscope show extensive hemorrhage, infiltration of lymphoid cells including neutrophils and macrophages, and edema in the alveolar spaces. Several studies have suggested that an overreaction of the host's immune system is involved in pathogenesis of influenza virus infection, and that morbidity and mortality are mediated as immunopathological consequences (*1–5*). Neutrophils and macrophages are known to produce superoxide free radicals (O_2^-) and hydrogen peroxide (H_2O_2), which normally are involved in the killing of ingested or invading microbes. However, the activated oxygen can also cause tissue injuries such as lung damage in adult respiratory distress syndrome and other inflammatory diseases (*6, 7*). Thus, it is possible that complex cellular immunity in influenza virus infection involves oxygen free radicals.

To test the hypothesis that free radicals are involved in influenza pathogenesis, we first studied O_2^- generation by the alveolar phagocytic cells from influenza virus–infected mice (Table 1). On day 8 after virus infection, the O_2^- generating potency of alveolar phagocytic cells was about eight times higher than that on day 0 (immediately after virus inoculation) with or without phorbol myristate acetate (PMA), a potent stimulant of O_2^- generation.

To see whether there was any increase in phagocytic cells in the lung, the population of lung-infiltrated cells was analyzed by broncho-alveolar lavage every other day after infection. On day 0 about 94% of the nucleated cells obtained by broncho-alveolar lavage were macrophages. Granulocytes in-

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