in estradiol 17β-dehydrogenase activity upon progestin administration (in E_2 controls, 4 nmole of E_1 was formed, but in progestin-treated animals, 10.1 nmole of E_1 was formed per milligram of protein per hour). A progestin-induced change in subnuclear vacuolization was observed only in histological sections of tumors of E_2-exposed animals treated with Depo-Provera.

Shafie (*2*) suggested that estrogen had an indirect action on tumor growth since the growth of MCF-7 cells was entirely dependent on E_2 in nude mice but was independent of estrogen in culture. Several other investigators (*3*) have previously proposed similar indirect mechanisms in tumor growth in other estrogen target tissues and have attributed this to the involvement of host-related factors, such as estromedins. 17β-Estradiol has also been shown to reduce natural killer cells in mice (*9*). If host factors, rather than receptor-mediated events, are solely responsible for the differential rate of growth of tumors in E_2-exposed nude mice, then one would expect a similar effect of E_2 on the growth of estrogen receptor negative as well as positive endometrial tumors. However, the present studies show a distinct difference in regard to E_2-dependent growth only in receptor-containing endometrial adenocarcinoma. As far as we are aware, this is the first direct demonstration of accelerated growth of primary tumors of human endometrium by E_2. We have recently suggested that endometrial carcinoma may result from aberrant endometrial cell differentiation (*10*). According to our proposed scheme the growth rate of only the receptor-positive endometrial tumors could be influenced by E_2. The results presented here confirm this prediction. Both of these tumors have been serially transplanted into nude mice five times with maintenance of their morphologic and biochemical characteristics.

Several epidemiologic studies have indicated a relation between the use of exogenous estrogens and an increased incidence of endometrial carcinoma. Our results suggest that E_2, by virtue of its ability to accelerate the growth rate of an already existing tumor, might lead to increased detection. The observation that estrogen use in postmenopausal women primarily increases the risk of development of endometrial cancers of early stage and low grade (*11*) supports such an interpretation.

Currently, there are no other model systems available for studying endometrial carcinoma and its hormonal regulation. The established endometrial cancer cell lines or organ culture systems either do not contain receptors for steroid hormones or are unresponsive to steroids added to the culture medium. Recently, we demonstrated that the lack of progestin sensitivity in cultured endometrial carcinoma explants in vitro is due to progesterone receptor instability under culture conditions (*8*). Since the steroid receptors are preserved in human endometrial carcinoma during serial transplantation with the maintenance of progesterone receptor synthetic machinery as well as sensitivity to progestin, the ovariectomized nude mice system appears to be an ideal model for studying steroidal and antisteroidal regulation of endometrial growth.

P. G. SATYASWAROOP
Department of Obstetrics and Gynecology, Milton S. Hershey Medical Center, Pennsylvania State University, Hershey 17033
R. J. ZAINO
Department of Pathology, Milton S. Hershey Medical Center
R. MORTEL
Department of Obstetrics and Gynecology, Milton S. Hershey Medical Center

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19 July 1982; revised 21 September 1982

Dimethyl Sulfoxide Stimulates Tyrosine Residue Phosphorylation of Rat Liver Epidermal Growth Factor Receptor

Abstract. *Epidermal growth factor, a potent mitogen, stimulates phosphorylation of its 170,000-dalton plasma membrane receptor. Dimethyl sulfoxide selectively increased phosphorylation of the epidermal growth factor receptor in rat liver microsomal fraction. Maximal stimulation occurred at 15 to 25 percent dimethyl sulfoxide and resembled the effect of epidermal growth factor in magnitude and rapidity. Like epidermal growth factor, dimethyl sulfoxide selectively stimulated tyrosine residue phosphorylation of this protein.*

A newly described class of protein kinases that selectively phosphorylate tyrosine residues has been linked to the regulation of normal and neoplastic growth because of the association of these kinases with viral transformation and with growth factor receptors (*1–6*). The transforming gene products of the Rous sarcoma virus and other RNA tumor viruses are protein kinases that phosphorylate tyrosine residues in cellular fractions (*2*) and in cultured cells (*3*). The activity of the enzyme is correlated with the appearance of the transformed phenotype. Nucleic acid hybridization studies have shown that the transforming retrovirus genes that code for these kinases are homologs of normal cellular genes (*4*).

Tyrosine residue kinase activity is also associated with growth factor receptors (*5, 6*). Epidermal growth factor (EGF), a mitogen in many tissues and cell lines (*7*), stimulates the phosphorylation of tyrosine residues in membrane proteins of a human epidermoid carcinoma line, A-431, and of several other cell types and tissues (*5, 8, 9*). One or two EGF-stimulated phosphoprotein bands of molecular weight 150,000 to 180,000 have been observed in each instance. The EGF-stimulated kinase and its substrate purify with the A-431 cell EGF receptor on affinity chromatography; the kinase and substrate are immunoprecipitated along with the EGF receptor by an antibody to the receptor (*10*). These findings indicate that the kinase and substrate are intrinsic to the EGF receptor. This complex could mediate EGF action by phosphorylating cellular proteins on tyrosine residues (*11*).

Hepatic DNA synthesis is stimulated by EGF in vitro and in vivo (*12*). We

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assessed the physiologic role of EGF and its receptor by studying EGF binding during rat liver regeneration. The number of EGF receptors decreased before the onset of DNA synthesis (*13*). EGF stimulated tyrosine residue phosphorylation of a single 170,000-dalton membrane protein (p170), which migrated on sodium dodecyl sulfate (SDS)–polyacrylamide gels along with the EGF receptor; the latter was identified by chemical cross-linking of ^{125}I-labeled EGF to membranes (*14*). During liver regeneration, EGF-dependent p170 phosphorylation decreased in parallel with the number of EGF receptors (*9*). Similar reductions in EGF binding are seen in a number of cell types treated with EGF or other growth factors (*15*). Thus, growth factors may be acting on the liver EGF receptor during physiologic growth.

During these studies, we observed that the polar solvent, dimethyl sulfoxide (DMSO), which has a number of unexplained effects on cultured cells, selectively stimulated p170 phosphorylation. The solvent induces differentiation of murine erythroleukemia (MEL) cells (Friend cells) (*16*), has various effects on growth and differentiated function in hepatoma cell lines (*17, 18*), and causes alterations in cell structure (*19*) and ion transport (*16*). We characterized the effect of DMSO on p170 phosphorylation and compared it to the effect of EGF.

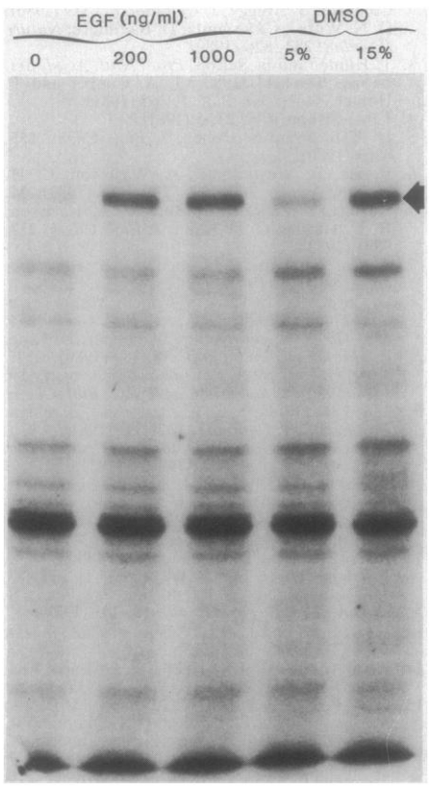

Fig. 1. Effect of EGF or DMSO on membrane protein phosphorylation. Liver microsomal fraction (50 μg of protein) was incubated for 10 minutes at 0°C in the presence of DMSO or EGF. The phosphorylation reaction (1 minute at 0°C) was initiated by adding 1 μ*M* [γ-^{32}P]ATP (10 μCi) to a final volume of 50 μl, containing a buffer (50 m*M* Pipes and 1 m*M* PO_4, *p*H 7.0) and 30 m*M* $MgCl_2$. The reaction was terminated by the addition of SDS and heating to 100°C for 3 minutes. A portion (37.5 μg of protein) was analyzed by electrophoresis and autoradiography. Both EGF and DMSO stimulate phosphorylation of a 170,000-dalton protein (p170, arrow).

Protein phosphorylation was studied by incubation of microsomal fraction (*20*) with [γ-^{32}P]adenosine triphosphate (ATP) at 0°C. Phosphoproteins were separated on SDS–polyacrylamide gels (8 percent polyacrylamide) and detected by autoradiography as described (*9*). The density of the autoradiographic bands was measured by densitometric scanning with on-line peak integration. Microsomal fraction was phosphorylated after incubation with EGF (200 or 1000 ng/ml) or with DMSO (5 or 15 percent). Some phosphorylation of p170 was observed in untreated membranes. Both compounds stimulated p170 phosphorylation in a concentration-dependent manner; the degree of maximum stimulation was similar (6.6- and 7.6-fold, respectively; Fig. 1). Phosphorylation of a 130,000-dalton protein was stimulated 50 percent by DMSO (15 percent) in this experiment, but this was not consistently observed. Phosphorylation of other proteins was generally unaffected by DMSO.

The response to a broader range of DMSO concentrations was also studied. At all concentrations tested (3 to 30 percent), DMSO stimulated p170 phosphorylation, with a maximum effect at 15 to 25 percent (Fig. 2). At a concentration of 20 percent, DMSO increased p170 phosphorylation by 14.3 ± 2.2-fold (mean ± standard error of four experiments, range 8.6 to 18.9). The DMSO-stimulated phosphorylation of p170 was as rapid as that produced by EGF; a substantial increase was detected at 15 seconds (0°C), the earliest time tested (not shown). The labeling of most other membrane phosphoproteins was inhibited at the higher (25 to 30 percent) concentrations of DMSO (not shown).

To study the phosphoamino acid specificity of the DMSO-stimulated kinase, we phosphorylated the microsomal fraction in the presence or absence of 20 percent DMSO. Protein was precipitated and hydrolyzed, and the phosphoamino acids were separated by two-dimensional thin-layer electrophoresis and chromatography (*3*). The plates were analyzed by autoradiography, and the spots were scraped and eluted for liquid scintillation counting. The abundance of phosphotyrosine was calculated as a proportion of total phosphoamino acid present. The phosphotyrosine abundance increased from 1.2 ± 0.2 to 5.3 ± 0.7 percent of the total, a statistically significant change ($N = 4$, $P < .001$ by paired *t*-test) (Fig. 3, A and B).

To determine whether the additional phosphotyrosine was present in p170, we phosphorylated membranes in the presence or absence of 20 percent DMSO; we isolated p170 by gel electrophoresis and analyzed it. Tyrosine residue phosphorylation of p170 was greatly increased (Fig. 3, C and D).

The DMSO-stimulated phosphorylation of p170 resembles that caused by EGF in its selectivity, magnitude, rapidity, and amino acid residue specificity. These findings indicate that DMSO, like EGF, activates the membrane EGF receptor-kinase-substrate complex. Dephosphorylation of p170 is very slow at 0°C (*21*), ruling out alterations in phosphatase activity. The kinase activation by DMSO presumably involves a conformational change in the EGF receptor. The activities of several other membrane-bound and soluble enzymes are affected by DMSO (*16, 22*). The activation could result either from an action of DMSO on the membrane [reduction of fluidity (*23*)] or from a direct effect on the protein.

Murine erythroleukemia cell differentiation is observed when low concentra-

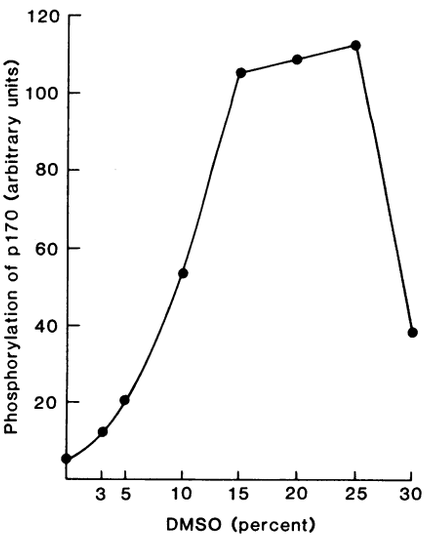

Fig. 2. Effect of various concentrations of DMSO on membrane protein phosphorylation. Liver microsomal fraction was phosphorylated in the presence of various concentrations of DMSO as described in Fig. 1. The p170 bands of the autoradiograms were analyzed with a densitometer (Perkin-Elmer) and integrator (Hewlett-Packard). DMSO stimulated p170 phosphorylation at all concentrations tested, with a peak at 15 to 25 percent (by volume).

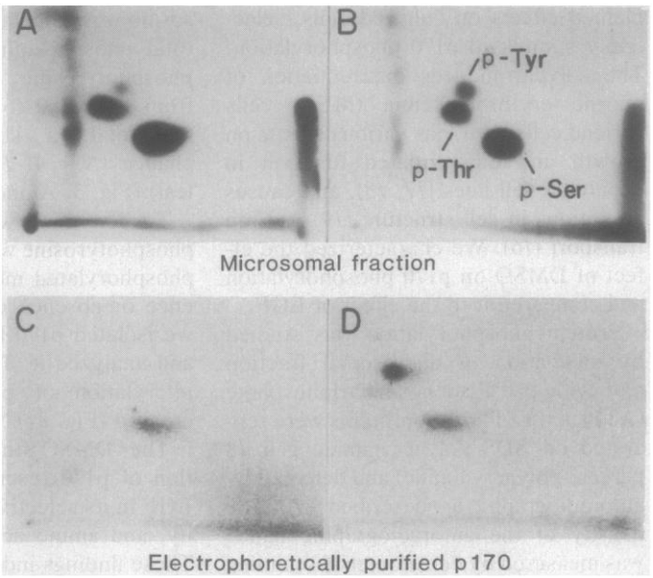

Fig. 3. [^{32}P]Phosphoamino acid content of membranes or p170 phosphorylated in the absence (A) and (C) or presence (B) and (D) of DMSO. In (A) and (B), liver microsomal fraction (1 mg of protein) was phosphorylated in the presence of 15 μM [γ-^{32}P] ATP (25 μCi) in 300 μl for 1 minute at 0°C. Trichloroacetic acid (TCA) was added to 50 percent, and the precipitate was washed with TCA (twice) and with a mixture of ether and ethanol (1:1) and dried. The samples were hydrolyzed in 6N HCl under N_2 for 3 hours at 110°C and lyophilized. The ^{32}P-labeled phosphoamino acids and added standards were separated by two-dimensional thin-layer electrophoresis (glacial acetic acid, 88 percent formic acid, and H_2O, 78:25:897 by volume, pH 1.9, at 900 V for 45 minutes) and ascending chromatography (isobutyric acid and 0.5M NH_4OH, 5:3 by volume) (3) and detected by autoradiography. Incorporation into p170 (C) and (D) was studied by phosphorylating the microsomal fraction (160 μg of protein) with 1 μM [γ-^{32}P]ATP (80 μCi) in 400 μl and subjecting it to SDS–polyacrylamide gel electrophoresis. The p170 was cut from stained gels and eluted by electrophoresis into dialysis bags. Bovine serum albumin (1 mg) was added, and the protein was precipitated with 15 percent TCA and analyzed. Phosphorylation of tyrosine residues was selectively stimulated by DMSO; phosphotyrosine (*p-Tyr*), phosphothreonine (*p-Thr*), and phosphoserine (*p-Ser*), respectively.

tions of DMSO (usually 1 to 3 percent) are present for 12 to 24 hours. While 3 percent DMSO increased p170 phosphorylation, maximum stimulation occurred at higher concentrations (15 to 25 percent) that are generally toxic to cells. Similarly, maximum phosphorylation of p170 is observed at concentrations of EGF much higher than those used to stimulate cell growth in culture. This suggests that there are many "spare receptors" for EGF, so that maximum binding and phosphorylation may not be necessary for a maximum cellular response. By analogy, low DMSO concentrations could affect intact cells by producing a submaximal but biologically effective stimulation of a tyrosine kinase. In addition, since basal phosphorylation of p170 occurs primarily on serine residues, a severalfold stimulation of tyrosine phosphorylation may appear as only a slight increase in total p170 labeling.

Some of the effects of DMSO on other membrane functions are analogous to known actions of EGF. Both EGF and DMSO alter ionic fluxes; Ca^{2+} uptake is stimulated in A-431 cells by EGF (*24*) and in MEL cells by DMSO (*25*). Uptake of Ca^{2+} is believed to be involved in the regulation of cell growth (*26*) and is necessary for the DMSO-induced differentiation of MEL cells (*25*). Properties of other cell surface receptors are also altered by DMSO; these effects include a reversible decrease in the affinity of the insulin receptor (*27*) and inhibition of the capping of lentil lectin receptors in *Dictyostelium* and surface immunoglobulin G on mouse lymphocytes (*28*). Changes in actin localization induced by DMSO, such as disruption of microfilaments, have been observed in several cell types (*19*).

Common actions of EGF and DMSO could indicate the significance of EGF receptor phosphorylation. Both EGF and intragastrically administered DMSO inhibit gastric acid secretion (*29, 30*). When administered repeatedly, EGF causes fatty infiltration of the liver (*31*), a phenomenon that also occurs during liver regeneration (*32*). Intraperitoneal administration of DMSO also causes fatty infiltration of the liver (*33*). In culture, DMSO stimulates the incorporation of [^{3}H]thymidine into the DNA of Novikoff hepatoma cells (*17*), but retards growth and leads to a more differentiated function in other hepatoma lines (*18*) and in MEL cells (*16*). Whether altered protein phosphorylation plays a role in these diverse actions of DMSO is not known. It will be interesting to see whether DMSO mimics other cellular actions of EGF, such as the potentiation of action of a newly identified class of EGF-dependent transforming growth factors (*34*).

The use of topical application of DMSO as a home remedy for arthritis and athletic injuries is increasing, and long-term hazards of repeated application remain to be determined. The DMSO is present in high concentration at the site of application and is rapidly absorbed. Since the EGF receptor is present in many tissues, DMSO could act at various sites. Although the significance of p170 tyrosine residue phosphorylation is unknown, its relation to growth factor action suggests that it may participate in growth stimulation. If DMSO induces inappropriate cellular proliferation in vivo, long-term toxicity might result.

RICHARD A. RUBIN*
H. SHELTON EARP
Cancer Cell Biology Program, Cancer Research Center, and *Department of Medicine, University of North Carolina, Chapel Hill 27514*

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20. Rat liver (1 g) is homogenized in 10 ml of ice-cold 0.25M sucrose and 10 mM tris, pH 7.4, for 30 seconds in a polytron (Brinkmann) set at 7.5. The homogenate is centrifuged at 30,000g (maximum) for 7.5 minutes. The supernatant is centri-

fuged at 137,000*g* (maximum) for 60 minutes, and the sediment (microsomal fraction) is resuspended in 1 ml of 20 m*M* Pipes, *p*H 7.0. Protein is determined by the method of O. H. Lowry, N. J. Rosebrough, A. L. Farr, R. J. Randall, [*J. Biol. Chem.* **193**, 265 (1951)].
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35. We thank K. Austin, R. Dy, and C. Edwards for technical assistance and K. Burridge, E. O'Keefe, and J. Pledger for reviewing this manuscript. This work was done while R.A.R. held postdoctoral fellowship 5T32 CA 90156 from the National Cancer Institute and during the tenure of an established investigatorship (H.S.E.) of the American Heart Association, with funds contributed in part by the North Carolina Heart Association. Supported by the American Cancer Society institutional grant IN-15W (R.A.R.), by NIH grant AM-30002 (H.S.E.), and by a grant in aid from the American Heart Association (H.S.E.).
* Send requests for reprints to the Department of Medicine.

18 May 1982; revised 5 October 1982

Oxidative Bioactivation of *S*-Alkyl Phosphorothiolate Pesticides: Stereospecificity of Profenofos Insecticide Activation

Abstract. *The mouse liver microsomal mixed-function oxidase system converts several phosphorothiolate pesticides with* S*-ethyl,* S*-propyl, or* S*-butyl groups to more potent inhibitors of acetylcholinesterase. This activation is stereospecific for the chiral isomers of* O*-(4-bromo-2-chlorophenyl)* O*-ethyl* S*-propyl phosphorothiolate (profenofos insecticide); the more toxic (−) isomer becomes a 34-fold better inhibitor of acetylcholinesterase in vitro, whereas the less toxic (+) isomer is deactivated by a factor of 2. Prior treatment of the microsomes with piperonyl butoxide or another mixed-function oxidase inhibitor markedly decreases the activation. Piperonyl butoxide also protects against brain acetylcholinesterase inhibition and cholinergic symptoms in chicks resulting from (−)-profenofos administration, thus establishing the importance of the oxidative bioactivation of* S*-alkyl phosphorothiolate pesticides in vivo.*

The phosphorothiolate (P–S–C) substituent is present in about 15 percent of the commercial insecticides and in many nematocides, herbicides, and fungicides. Insecticides with the phosphorothionate (P = S) substituent, such as parathion, require oxidative bioactivation before inhibiting acetylcholinesterase (AChE) (*1*). The possibility of a comparable bioactivation of phosphorothiolates have eluded experimental verification (*1–3*). Two recent findings with the insecticide profenofos (*4*) [*O*-(4-bromo-2-chlorophenyl) *O*-ethyl *S*-propyl phosphorothiolate] (compound **4** in Table 1) have prompted renewed interest in possible bioactivation of phosphorothiolates. First, it was surprising to find that the (−) isomer of profenofos is the more toxic to mice and each of three insect species assayed, whereas the (+) isomer is the more potent in vitro inhibitor of four esterases including AChE (*5*). This finding suggests differential detoxification of the isomers or, more likely, that the (−) isomer undergoes bioactivation, whereas the (+) isomer is unaltered or detoxified under the same conditions. Second, peracid oxidation converts profenofos into a potent phosphorylating agent, which is proposed to be the phosphorothiolate *S*-oxide (*6*). We now provide definitive evidence—including appropriate stereospecificity for profenofos—for oxidative bioactivation of various phosphorothiolate pesticides (Fig. 1).

A simple detoxification or bioactivation assay for AChE inhibitors (*7*) was modified for the analysis of unstable metabolites formed by the mixed-function oxidase (MFO) system. Activation on NADPH (reduced nicotinamide adenine dinucleotide phosphate) fortification is evident for parathion and the *S*-ethyl, *S*-propyl, and *S*-butyl phosphorothiolates, but not for the *S*-methyl and *S*-benzyl phosphorothiolates (Table 1). Lowering the phosphorothiolate concentrations results in decreased AChE inhibition after activation. The activation of (±)-profenofos and the *S*-ethyl isomer of *O,O*-diethyl *O*-phenyl phosphorothionate (SV-1) is strongly inhibited by piperonyl butoxide (PB) and SV-1 (Table 1), two MFO inhibitors that act by entirely different mechanisms (*8*). Compounds for which activation is not detected at $10^{-4}M$ include methamidophos and Kitazin P (Table 1) and *O,S*-dimethyl acetylphosphoramidothioate (acephate).

The profenofos chiral isomers (*5*) react in an opposite manner in the oxidase system in vitro (Fig. 2). (−)-Profenofos is activated by a factor of 34, whereas (+)-profenofos is deactivated by a factor of 2. Although (+)-profenofos is intrinsically the more potent AChE inhibitor, (−)-profenofos on activation becomes even more effective than (+)-profenofos. These findings are consistent with the greater toxicity of the (−) isomer relative to the (+) isomer (*5*). The reversal of chiral specificity in the bioactivation system may be due to differences in rates of oxidation or, more probably, in the enantiomeric selectivity of AChE inhibition (*1, 9*). Thus AChE reacts preferentially with the nonoxidized form of (+)-profenofos and the oxidized form of (−)-profenofos. This may involve a change in the leaving group, that is, cleavage of the P–O–phenyl linkage for (+)-profenofos and of the activated P–S–propyl linkage for (−)-profenofos (Fig. 1) (*10*).

(−)-Profenofos also undergoes MFO

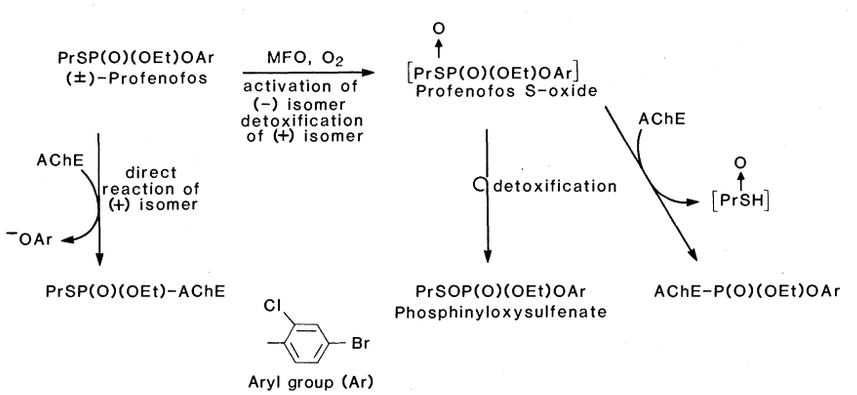

Fig. 1. Proposed mechanism for reversal of chiral specificity for acetylcholinesterase inhibition after oxidation of profenofos enantiomers.

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