# The Contribution of Tobacco Constituents to Phenol Yield of Cigarettes[1]

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Phenol has been determined as a component of cigarette smoke for many years (2, 4, 5, 6, 8, 9), but until recently no studies have appeared on the origin of phenol with respect to specific tobacco constituents, although some investigators have speculated in this area.

It would appear possible to study the conversion of tobacco constituents during the smoking process by enriching or depleting tobacco with respect to a certain constituent. However, this introduces a number of variables which are difficult, if not impossible, to control. Addition of significant amounts of material—five to ten percent by weight—is usually necessary to bring about demonstrable changes in smoke composition, and, undoubtedly, the burning characteristics of the tobacco are changed. Extraction of tobacco with solvents to deplete certain constituents produces similar alterations in the burning process.

The pyrolytic technique has been used by several investigators to simulate the burning characteristics of tobacco constituents and to study the yield of smoke components (3, 10), but only one investigation has dealt directly with the precursors of phenols in tobacco smoke (1). Pyrolysis experiments are convenient to study the thermal conversions to tobacco constituents, but unless the conditions which exist in the burning cigarette during a specific conversion can be approximated, pyrolytic studies may be misleading. Moreover, when individual compounds are pyrolyzed, the absence of other tobacco components cannot be neglected in reaching conclusions, since they may produce catalytic effects.

The addition of $C^{14}$ labeled compounds to tobacco appears to offer the most unequivocal technique for studying the contribution of precursors to smoke constituents. The labeled compounds can be added to tobacco in extremely small quantities, and no difficulty is experienced in obtaining cigarettes with normal burning characteristics.

In this investigation of phenol precursors the pyrolytic method was adopted to develop preliminary conclusions, and then the tracer technique used to substantiate or refute these conclusions.

## Experimental Methods

### *A. Pyrolytic*

The pyrolysis apparatus shown in **Figure 1** consisted of a 100 cm x 2.5 cm Vycor tube packed to about 60 cm with Vycor chips. The packed end of the tube was heated independently of the sample end by two standard combustion furnaces. The sample was placed in a Pyrex tube with an external diameter slightly less than the internal diameter of the combustion tube and located about 15 cm from the Vycor packing. A third furnace was driven mechanically over the sample toward the fixed furnaces. Air or nitrogen was passed through the combustion tube at a fixed rate. All temperatures were controlled within 3°C. The trapping system consisted of a conventional cold trap submerged in a dry ice-acetone slurry. Phenol analyses were carried out by the method described by Spears (9), except the steam distillation was omitted. The relative standard deviation of the pyrolytic procedure was found to be 10%.

### *B. Tracer*

The radioactivity was measured by liquid scintillation counting. All samples were placed in 13 to 17 ml of the scintillating solution composed of toluene containing 6 g/liter 2,5-diphenyloxazole and 0.10 g/liter 1,4-bis(2-[5-phenyloxazolyl])-benzene and counted at 0°C. Quenching was determined by the method described by Ross (7).

Approximately four grams of tobacco were treated with 50 ml of an ethanol-water solution containing $5.12 \times 10^5$ cpm/ml of uniformly labeled glucose [specific activity 200 mc/mM]. After thorough mixing, the solvent was evaporated on a rotating evaporator and then lyophilized to ensure that all solvent was removed. The dried tobacco was removed from the flask and the flask washed several times with the ethanol-water solution. These washings were combined with the trapped solvent evaporated from the tobacco and the radioactivity measured. By subtracting this value from the counts



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# STF – IL-4: a novel IL-4-induced signal transducing factor

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**The mechanism by which interleukin-4 (IL-4) regulates the expression of particular genes is unknown. We have determined that IL-4 induces a DNA binding factor (termed STF – IL-4) which has a strong affinity for an IFN-γ activation site (GAS). Interestingly, STF – IL-4 also binds to the IL-4 responsive promoter for the Ig heavy chain germline ε transcript. The IL-4 dependent activation of STF – IL-4 is rapid, does not require protein synthesis and results in the sequential appearance of binding activity first in the cytoplasm and then later in the nucleus. Activation of STF – IL-4 is sensitive to tyrosine kinase inhibitors and the active factor is tyrosine phosphorylated. This pattern of activation is similar to the activation of interferon-induced transcription factors. STF – IL-4 appears to be a new member of a growing family of cytokine-induced transcriptional regulators.**
*Key words:* cytokine/interferon/interleukin-4/signal transduction

## Introduction

Interleukin-4 (IL-4), which is produced by T cells, mast cells and basophils, has potent biological activities on many different cells, including B and T lymphocytes, mast cells and macrophages (reviewed in Spits, 1992). When cells are cultured with IL-4, it often stimulates a variety of different functions within a given cell type. For instance, IL-4 can stimulate B cells to proliferate and differentiate (reviewed in Paul and Ohara, 1987), alter the level of cell surface receptors such as MHC class II (Noelle *et al.*, 1984; Roehm *et al.*, 1984) and CD23 (Defrance *et al.*, 1987; Hudak *et al.*, 1987), and stimulate the B cells to undergo Ig heavy chain class-switching to IgG1 and IgE (reviewed in Coffman *et al.*, 1993). Many of these biological activities rely on IL-4's ability to stimulate the transcription of particular genes. For instance, the ability of IL-4 to stimulate Ig heavy chain class-switching to IgG1 and IgE is dependent on its ability to stimulate germline γ1 and ε transcription prior to switching (reviewed in Coffman *et al.*, 1993).

The gene encoding the IL-4 cellular receptor has been cloned (reviewed in Izuhara *et al.*, 1993) and is a member of the hematopoietin receptor superfamily (Cosman, 1993). The gene encodes a 140 000 kDa molecule which contains a conserved pattern of cysteine residues as well as a WSXWS box, which are found in other members of this superfamily of cell surface receptors (Miyajima *et al.*, 1992). In addition, the IL-4 receptor does not contain recognizable kinase domains. Although the ability of IL-4 to induce the transcription of certain genes has been well documented, the mechanism by which the binding of IL-4 to its receptor stimulates transcription of these genes remains unknown. Murine IL-4 does not seem to activate a cell through the mobilization of $Ca^{2+}$ or by activation of the phosphoinositol pathway (Justement *et al.*, 1986; Mizuguchi *et al.*, 1986).

Recently, the pathway by which the interferons (IFNs) stimulate transcription of early response genes has been elucidated (reviewed in Pellegrini and Schindler, 1993). The binding of IFN (either α/β or γ) to its receptor stimulates the activation by tyrosine phosphorylation, of latent cytoplasmic transcription factors, termed signal transducing factors (STF) – IFN-α/β or – IFN-γ, respectively. After this phosphorylation event, these factors translocate to the nucleus where, after binding to specific DNA elements [ISRE and the IFN-γ activation site (GAS) respectively] in the promoter of responsive genes, they function as transcriptional activators. The components of these STF complexes have been purified and cloned, and appear to represent a novel class of transcriptional activators. One of these proteins, p91, is a component of both STF – IFN-α and STF – IFN-γ (reviewed in Pellegrini and Schindler, 1993).

This system of signal transduction is important in the biological response to IFNs which require the rapid activation of specific target genes. It appears that other cytokines may employ a related signaling paradigm. For instance, both CNTF (Bonni *et al.*, 1993) and EGF (Sadowski *et al.*, 1993) rapidly activate STFs after binding to their respective receptors. Interestingly, the p91 protein appears to be a component of both STF – CNTF and STF – EGF (Bonni *et al.*, 1993; Silvennoinen *et al.*, 1993a,b). Recent evidence indicates that other cytokines also induce STFs and that several of these STFs bind to a site in the promoter of the *IRF-1* (ISGF2) gene (termed IRF-1 GAS) which has been shown to bind STF – IFN-γ (Pine *et al.*, 1994; P.Rothman and C.Schindler, unpublished observations).

IL-4 and IFN-γ are produced by different subsets of $CD4^+$ helper T cells (reviewed in Mosmann and Coffman, 1989). These two cytokines regulate several steps in B cell development. One model by which two different cytokines could regulate the transcription of the same genes would involve similar pathways of signal transduction. In an effort to characterize a putative STF that could mediate the effects of IL-4 on B cell development, we determined that IL-4 rapidly activates a novel STF which has an affinity for the GAS element. The activation and activity of this factor (termed STF – IL-4) share many biological features with the IFN pathway. In addition, STF – IL-4 binds to a sequence at the IL-4 inducible promoter of the Ig germline ε transcript that differs significantly from the GAS element.

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# Determination of Tetrahydroisoquinoline Alkaloids in Biological Materials with High Performance Liquid Chromatography

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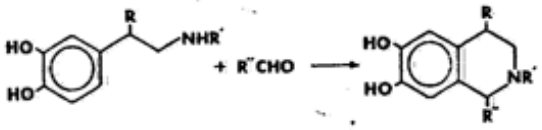
**A new approach is described for the selective determination of tetrahydroisoquinolines (TIQ's) formed by condensation of catecholamines with aldehydes in vivo. High performance cation exchange chromatography in combination with amperometric detection permits simultaneous quantitation of tetrahydropapaveroline (THP) and salsolinol (SAL) at 2 ng/g in rat brain and 2 ng/mL in urine. Examples of application of the new method are given for studies pertaining to the possible involvement of TIQ's in alcohol abuse.**

The primary metabolic pathways of the catecholamines have been extensively studied and their involvement in neurotransmission and other processes in the peripheral and central nervous systems has long been recognized. There exists, however, another possible metabolic route for these compounds which has only recently received attention. This pathway involves the nonenzymatic condensation of catecholamines with aldehydes to form tetrahydroisoquinoline alkaloids (TIQ's) which can act as "false" neurotransmitters and result in catecholamine depletion. Such a reaction scheme is shown below.




Although it is conceivable that this reaction could occur normally by utilizing endogenous aldehydes (e.g., glyceraldehyde or glyoxylate), these compounds are generally present at low concentrations within the mitochondria or other organelles of the cell and thus never encounter the catecholamines. TIQ formation is believed to have its most probable significance under abnormal circumstances in which the body is presented with an external stimulus which results in significant levels of circulating aldehydes. The most obvious example of such a stimulus is alcohol consumption. During alcohol intoxication, significant levels of circulating acetaldehyde are present and it has been proposed by various workers that the condensation of acetaldehyde with catecholamines to form TIQ's may account in some way for the addicting properties of alcohol. The literature adequately describes the rationale for this hypothesis (*1–4*). It appears as though no definite conclusions have been reached as to the credibility of this idea; however, the balance of evidence reported to date is negative. A recent encouraging report by Myers and Melchior indicates that drinking behavior in rats can be influenced by ventricular perfusion of TIQ's in brain (*5*). There are also reports that TIQ's form during various drug therapies (e.g., L-Dopa treatment of Parkinson's disease) and may play some role in the pharmacological effects of these drugs (*6*).

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It has long been recognized that available analytical methods for catecholamines are quite poor, especially when dealing with endogenous levels in biological samples. Since TIQ levels will always be significantly less than their catecholamine precursors, it is obvious that their assay will be even more difficult. This is indeed the case and has hampered workers in this area to a large extent.

Recently we have described a simple and sensitive assay procedure for urinary and tissue catecholamines (*7, 8*). This method utilizes high performance liquid chromatography (HPLC) coupled to a thin-layer electrochemical detector (LCEC). The advantages of using liquid chromatography as opposed to gas–liquid and other forms of chromatography or fluorometry to assay catecholamines are obvious and have been adequately described by us and others (*7, 9*). The same advantages apply to the present TIQ assay. The fundamental problem with HPLC analysis of TIQ's is the relatively poor sensitivity of the commonly used detectors (e.g., UV-VIS absorption). The use of thin-layer electrochemistry as a detection system has numerous advantages in terms of sensitivity, selectivity, and cost. The sensitivity using electrochemical detection can sometimes be two to three orders of magnitude better than for UV and its inherent selectivity makes it a much more feasible approach for assays of many biological materials.

A fundamental advantage (in many cases a limitation) of LCEC is the fact that only electroactive compounds can be detected. For assay of TIQ's, this turns out to be a great advantage since all the compounds of interest contain at least one phenolic substituent, thus making them electrooxidizable. Figure 1 illustrates the electrochemical behavior of two typical TIQ's. By selecting a detection potential in the range of +0.7 to +0.8 V, one can oxidize the TIQ's (and catecholamines) while not detecting compounds with much larger oxidation potentials.

Using the LCEC technique, we have detected the presence of TIQ's in plant matter and in the urine of individuals following consumption of bananas or cocoa-based products (*10, 11*). This report describes analytical procedures for ppb levels of TIQ's in body fluids and tissues. Several preliminary studies on TIQ formation in vivo are also discussed.

## EXPERIMENTAL

The present assay for urinary TIQ's is a modification of our earlier procedure for catecholamines (*7*). Urine was collected over acid (to pH 2) using 6 M HCl and stored at −35 °C prior to analysis. Four milliliters of acid-hydrolyzed (*7*) urine were placed in polyethylene centrifuge tubes with 1.5 g of $(NH_4)_2SO_4$, capped and shaken, and centrifuged to remove solids and precipitated proteins. Acid hydrolysis is necessary to release the alkaloids from sulfate and glucuronide conjugation. The supernate was transferred to 12-mL glass centrifuge tubes and extracted twice with ethyl acetate and once with hexane (5-mL aliquots). The nonaqueous layers were discarded. The aqueous layer was transferred to a 10-mL beaker with 100 μL each of 5% sodium metabisulfite and 10% EDTA. The stirred sample was then adjusted to pH 8.5 and placed in a 5-mL conical vial containing 80 mg of alumina (*12*) and shaken for 12 min on a reciprocating shaker. The urine was aspirated off and the alumina was washed three times with distilled water and dried in vacuo for 3 min at 30 °C.

The compounds were eluted from the alumina with 400 μL of 1 M

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# Cigarette Smoke-Induced Depression in LCAT Activity

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The effect of acute inhalation of cigarette smoke on plasma cholesterol esterification by lecithin–cholesterol acyltransferase (LCAT) in atherosclerosis-susceptible White Carneau pigeons was examined. Pigeons were assigned to four treatment groups. (1) Shelf Control fed a chow diet and not exposed to smoke products. (2) Sham pigeons fed a cholesterol-saturated fat diet and exposed to fresh air by the Lorillard smoking machine. (3) low nicotine–low carbon monoxide (LoLo) animals also fed the cholesterol diet and exposed to low concentrations of these cigarette smoke products; and (4) high nicotine–high carbon monoxide (HiHi) birds fed the cholesterol diet and subjected to high concentrations of these inhalants. Both Control and Sham birds had significantly higher LCAT activity (percentage esterification per minute) than HiHi pigeons. Experiments designed to determine whether altered enzyme and/or substrate were responsible for depressed activity revealed no smoke-related modification in substrate efficiency. In addition, Sham and HiHi pigeons had similar concentrations of plasma-free cholesterol, high density lipoprotein (HDL) cholesterol, cholesteryl ester and phospholipid, and similar HDL phospholipid and cholesteryl ester fatty acid profiles. However, reduced LCAT activity in HiHi pigeons can be explained by (1) impairment of enzyme efficiency as estimated by *in vitro* analysis, and (2) *in vivo* reduction in levels of LCAT cofactor, HDL apoprotein A-I.

## INTRODUCTION

Lecithin–cholesterol acyltransferase (LCAT) (EC 2.3.1.43) is a plasma enzyme which catalyzes esterification of cholesterol on high-density lipoproteins (HDL) by transferring a fatty acid from the C-2 position of phosphatidyl choline to free cholesterol (Glomset, 1968). HDL apoprotein A-I is an important cofactor in this reaction (Fielding and Fielding, 1971; Soutar *et al.*, 1975). Although the metabolic significance of the LCAT reaction has not been completely elucidated, Glomset (1979) has postulated that LCAT may be involved in the removal of excess phospholipid and free cholesterol from lipoprotein remnants and may participate in the mobilization of cholesterol from peripheral cell membranes and its subsequent transport to the liver.

HDL serves as the lipoprotein substrate for LCAT and may also have antiatherogenic properties related to its ability to remove cholesterol from extrahepatic tissue (Glomset and Norum, 1973; Glomset, 1979). Numerous epidemiological studies have demonstrated a strong inverse relationship between HDL cholesterol concentration and the incidence of coronary heart disease (Gordon *et al.*, 1977; Castelli *et al.*, 1977). A similar correlation also exists between coronary heart disease and HDL's major protein component, apoprotein A-I (Berg *et al.*, 1976). Much recent attention has focused on HDL levels and established heart disease risk factors such as cigarette smoking (Enger *et al.*, 1977; Hulley *et al.*, 1979; Berg *et al.*, 1979; Garrison *et al.*, 1978). Results from these studies show that smoking reduces HDL cholesterol (Enger *et al.*, 1977; Hulley *et al.*, 1979; Garrison *et al.*, 1978) and apoprotein A-I and A-II levels (Berg *et al.*, 1979).



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