yields a pattern of very sharp DNA bands.[83] The molecular weights of these bands correspond very closely to integral multiples of about 135 base pairs, with perhaps a small "spacer" of about 10 base pairs.

As to the details of structural organization, we can only speculate at the moment. Van Holde et al.[84] have proposed a model in which the globular C-terminal portions of the histones aggregate to form a core, about which the DNA is wrapped. The projecting N-terminal basic regions are then assumed to wrap into the major groove of the DNA, holding it to the core.

The idea that much of the DNA is wrapped on the outside of the subunit gains credence from the results obtained by Noll[85] with DNAse I, in which digestion to fragments which are multiples of 10 base pairs was found. Apparently the DNA in the particles is at least partially accessible to some nucleases.

It is clear that at this point our views of the structure of chromatin are changing rapidly. The next few years should be interesting.

The work of K. E. Van Holde was supported by NSF Grant GB-37307X; that of I. Isenberg was supported by U. S. Public Health Service Grant CA-10872. We thank Dr. C. L. F. Woodcock for permission to use an unpublished electron micrograph. Drs. Martinson and McCarthy permitted the authors to read their paper prior to publication, and we thank them.

(83) D. Lohr and K. E. Van Holde, *Science*, 188, 165 (1975).
(84) K. E. Van Holde, C. G. Sahasrabuddhe, and B. R. Shaw, *Nucleic Acids Res.*, 1, 1579 (1974).
(85) M. Noll, *Nucleic Acids Res.*, 1, 1587 (1974).

Antibodies: Analytical Tools to Study Pharmacologically Active Compounds

John J. Langone, Helen Van Vunakis, and Lawrence Levine*

Department of Biochemistry, Brandeis University, Waltham, Massachusetts 02154

Received February 7, 1975

Competitive protein binding assays currently are being used to quantitate many different types of compounds.[1] They depend upon the affinity of a biological macromolecule for a particular ligand (e.g., intrinsic factor for vitamin B_{12}, thyroxine-binding globulin for thyroxine, a tissue receptor for a hormone, or an antibody for an antigen). Their sensitivity is made possible through the use of radioactive tracer molecules of high specific activity. The extent to which unlabeled ligand competes with radioactive ligand for a limited number of receptor sites serves as a basis for quantitation in this type of assay, and often picogram amounts of a particular compound can be estimated. Once the receptor molecule and labeled ligand are available, the assays are relatively simple to perform: mixtures containing labeled ligand, receptor molecule, and sample are incubated, free labeled ligand is separated from receptor-bound labeled ligand, and the extent of binding is determined.

Although natural receptors have been employed in several competitive binding assays, their use is limited by their availability and stability. The organic chemist, however, by designing proper antigens, often can produce antibodies in experimental animals that have the specificity and affinity required of a receptor molecule.

Radioimmunoassay (RIA). *Antibodies* are proteins found in the globulin fractions of blood that are produced by vertebrates in response to the presence of an *antigen,* i.e., a substance that is recognized by the host to be foreign.[2] Although antibodies are generally regarded as defensive weapons elicited for protection against invasion by pathogenic microorganisms, they also can be produced against specific low molecular weight compounds if these are covalently linked to macromolecules. Antibodies can show a remarkable ability to bind selectively the antigen that stimulated their production. Their *specificity* may be regarded as comparable to that of an enzyme for substrate. The binding constants between antibody and antigen are often of the order of 10^7 to 10^9 l./mol. This ability of antibodies to discriminate between the homologous antigen and the myriad of other compounds of widely diverse structure that are found in biological fluids, e.g., serum or urine, is of fundamental importance in their use as analytical tools.

The principle of the RIA technique was described by Berson and Yalow in 1960[3] (Figure 1). Constant amounts of free labeled antigen (Ag*) and a limited

John J. Langone is Senior Research Associate in the Biochemistry Department at Brandeis University. He received his Ph.D. degree in 1972 from Boston University in organic chemistry. His current interests include bioorganic chemistry, immunopharmacology, and immunochemistry of biologically active drugs.

Helen Van Vunakis is Professor of Biochemistry at Brandeis University. She received her Ph.D. degree in biochemistry at Columbia University, then held a postdoctoral position in the Department of Biochemistry at Johns Hopkins University, School of Hygiene and Public Health. Her research interests include structure and functions of proteins and nucleic acids, and immunochemistry and biochemistry of pharmacologically active compounds.

Lawrence Levine is Professor of Biochemistry at Brandeis University. He obtained a Sc.D. from the Johns Hopkins University, School of Hygiene and Public Health. His research utilizes immunochemical procedures for the study of biological problems.

(1) "Principles of Competitive Protein Binding Assays", W. E. O'Dell and W. H. Daughaday, Ed., J. B. Lippincott Co., Philadelphia, Pa., 1971.
(2) K. Landsteiner, "The Specificity of Serological Reactions", Harvard University Press, Cambridge, Mass., 1945; (b) E. A. Kabat, "Structural Concepts in Immunology and Immunochemistry", Holt, Rinehart and Winston, New York, N.Y., 1968; (c) H. N. Eisen, "Immunology", Harper and Row, New York, N.Y., 1974.

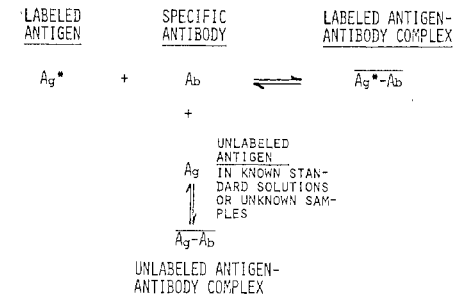


Figure 1. Principle of radioimmunoassay.

number of antibody sites (Ab) are in equilibrium with the labeled antigen–antibody (Ag*–Ab) complex. If unlabeled antigen (Ag), either in the standard solution or in the sample to be analyzed, is added, it competes for the limited number of Ab sites, and depending on its concentration displaces different amounts of the labeled Ag* from the Ag*–Ab complex.

RIA's have been developed for many different classes of compounds.[1,3–5] Macromolecules such as proteins, nucleic acids, and polysaccharides usually can elicit an immune response when they are injected into the experimental animal directly or in the form of an electrostatic complex. However, low molecular weight compounds ordinarily cannot elicit an immune response unless they are bound covalently to an antigenic macromolecule (e.g., protein or polypeptide).[2] Such low molecular weight compounds are called *haptens*.

Most pharmacologically active molecules are of low molecular weight. Therefore, some special problems concerned with (a) the synthesis of suitable covalent conjugates for immunization, (b) the preparation of radioactive ligands, and (c) the determination of serologic specificity of ligand–antibody binding will be discussed in this Account.

Synthesis of Covalent Conjugates for Immunization. Carboxy or amino functionalized haptens can be coupled directly to the amino or carboxy groups of amino acid residues in proteins or polypeptides by the use of a dehydrating agent such as a carbodiimide (CDI). This procedure, which leads to amide bond formation, and several others, including those that have been used to functionalize keto or hydroxy groups in haptens and to couple aldehydes directly, have been reviewed.[1,4–6] In cases where the hapten derivative must be synthesized from smaller molecules, e.g. RIA's for nicotine and cotinine,[7] the stereochemistry of the hapten must be considered and the syntheses should lead to a derivative in which the structural features of the parent molecule are preserved. Since the specificity of an Ab usually is directed toward those structures on the hapten that are distal to the linkage group, the hapten should be coupled to the carrier so that characteristic functional groups are exposed to antibody synthesizing cells.

The conjugates can be injected into experimental animals along with substances that will increase the immune response (*adjuvants*).[2] Various routes and schedules of immunization which have proven successful in eliciting the production of antibodies have been described.[2]

Synthesis of Radiolabeled Derivatives ^{3}H-, ^{14}C-, ^{125}I-, and ^{131}I-labeled compounds of high specific activity commonly are used to develop sensitive RIA's. Iodination of proteins and haptens which bear a primary or secondary amino group can be accomplished by acylation with the iodinated *N*-hydroxysuccinimide ester of *p*-hydroxyphenylpropionic acid.[8a] In the two commonly used iodination methods, the Chloramine T method[8b] and the enzymatic reaction catalyzed by lactoperoxidase,[8c,d] the presence of a phenolic group that can be substituted at the ortho position(s) is required. Since most haptens lack a phenolic group, methods to prepare phenolic derivatives of haptens suitable for iodination have been devised. These include coupling the hapten (or a suitable derivative) to polyvalent molecules such as proteins or copolymers that contain tyrosine, or to monovalent compounds that have a phenolic group.[4,7,9] However, polyvalent labeled derivatives tend to bind more strongly to antibody molecules due to stable lattice formation. Therefore, they are less satisfactory than their monovalent counterparts since the unlabeled antigen (contained, for example, in the sample to be analyzed) is less able to compete for antibody binding sites and the sensitivity of the assay often is diminished.[10]

Serologic Specificity of Ligand–Antibody Binding. Antibody concentrations appropriate for competition experiments are determined by measuring the ability of different dilutions of the antiserum to bind the labeled hapten or hapten derivative.[1,3,4] To determine the *sensitivity* of the assay, the percent inhibition at different concentrations of the homologous antigen is obtained, and a standard curve is constructed.

With monovalent Ag*, the hapten–antibody complexes and the free components are in equilibrium. To determine binding, free Ag* and antibody-bound Ag* (i.e., Ag*–Ab) are separated by procedures that will not disturb the equilibrium. Several methods, including precipitation of the antibody with a second antibody or with reagents such as $(NH_4)_2SO_4$, adsorption of free hapten to charcoal or other insoluble material or solid-phase (immobilized) antibodies, electrophoresis, chromatography, and filtration, have been used for this purpose.[1,3,4,10] By measuring the ability of different amounts of structurally related compounds to inhibit the homologous antigen–anti-

(3) (a) R. S. Yalow and S. A. Berson, *J. Clin. Invest.*, **39**, 1157 (1960); (b) S. A. Berson and R. Yalow in "Immunology", R. A. Good and A. W. Fisher, Ed., Sinauer Associates, Inc., Stamford, Conn., 1971; (c) R. S. Yalow, Suppl. 1 to *Circ. Res.*, **32–33**, 1–116 (1973).

(4) "Radioimmunoassay and Saturation Analysis," *Brit. Med. Bull.*, **30**, No. 1 (1974).

(5) V. P. Butler, Jr., and S. M. Beiser, *Adv. Immunol.*, **17**, 255 (1973).

(6) B. F. Erlanger in "New York Heart Association Symposium on Immunopharmacology", Williams and Wilkens, Baltimore, Md., 1973, p 281.

(7) J. J. Langone, H. B. Gjika, and H. Van Vunakis, *Biochemistry*, **12**, 5025 (1973).

(8) (a) A. E. Bolton and W. M. Hunter, *Biochem. J.*, **133**, 529 (1973); (b) F. Greenwood, W. M. Hunter, and J. Glover, *ibid.*, **89**, 114 (1963); (c) M. Morrison, G. S. Bayse, and R. G. Webster, *Immunochemistry*, **8**, 289 (1971); (d) J. I. Thorell and B. G. Johansson, *Biochim. Biophys. Acta*, **251**, 303 (1971).

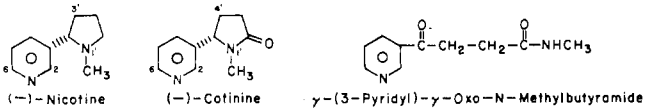
(9) (a) A. R. Midgley, Jr., G. D. Niswender, and J. Sri Ram, *Steroids*, **13**, 731 (1969); (b) W. T. Newton, J. E. McGuigan, and B. M. Jaffe, *J. Lab. Clin. Med.*, **75**, 886 (1970); (c) T. L. Goodfriend and D. L. Ball, *ibid.*, **73**, 501 (1969).

(10) H. Van Vunakis and L. Levine in "Immunological Assays of Drugs of Abuse", S. J. Mulé, I. Sunshine, M. Braude, and R. E. Willette, Ed., Chemical Rubber Publishing Co., Cleveland, Ohio, 1974.

Table I
Inhibition of Nicotine, Cotinine, and γ-(3-Pyridyl)-γ-oxo-*N*-methylbutyramide (Oxo Amide) Antigen–Antibody Reactions[7,13]

Compound	nmol required for 50% inhibition		
	[^{3}H]Nicotine–antinicotine	^{125}I-cotinine derivative–anticotinine[a]	^{125}I-oxo amide derivative–anti-oxo amide[b]
(−)-Nicotine	0.02	12.7	111
(−)-Cotinine	>50[c]	0.0067	23
γ-(3-Pyridyl)-γ-oxo-*N*-methylbutyramide	>25[c]	>20[c]	0.026
Nicotine *N'*-oxide	3.7	44.9	56
6-Hydroxynicotine	100	>25[d]	>50[d]
(−)-Nornicotine	2.2	67.5	23
trans-3'-Hydroxycotinine	>50[d]	0.095	130
dl-Desmethylcotinine	>50[d]	2.2	9.9
(−)-Cotinine *N*-oxide	>50[c]	6.2	>52[c]
γ-(3-Pyridyl)-γ-oxobutyric acid	200	>25[d]	3.35

[a] Labeled derivative is *N'*-(*p*-hydroxyphenethyl)-*trans*-4'-cotininecarboxamide. [b] Labeled derivative is γ-(3-pyridyl)-γ-oxo-*N'*-(*p*-hydroxyphenethyl)butyramide. [c] 20% inhibition at this level. [d] 10% inhibition at this level.



body reaction, the *specificity* of the antibodies is determined. From this information, the structural features of the hapten that are complementary to the antibodies' combining sites can be established.

RIA Compared to Other Analytical Methods. RIA is a highly sensitive, remarkably specific technique that is simple to perform and is adaptable to rapid analysis of large numbers of samples. Thus, it can offer distinct advantages over other analytical procedures. Chromatographic methods including paper, thin layer (TLC), and gas–liquid chromatography (GLC) are among the most widely used analytical tools to study the metabolism and disposition of biologically important molecules,[11] especially when labeled compounds are available to aid in the identification and for quantitation of the parent compound and its metabolites. GLC is particularly effective when combined with mass spectrometry.[11] With such systems, sensitivity down to the picomole level is not uncommon. High-pressure liquid chromatography (HPLC) is relatively new but is becoming an increasingly important tool to separate drugs and other molecules present in physiological fluids.[12] However, these chromatographic methods normally require that samples be extracted, distilled, or otherwise processed to concentrate the drug or metabolites and to separate them from endogenous constituents that might interfere with the assays. The severe conditions required by some of these techniques prevent their use with labile compounds.

Spectral techniques also are used,[11] but preliminary fractionation of biological samples may be necessary to separate the target compounds from other components that have similar spectral characteristics or that may change the spectrum by interacting with the compound.

The sensitivity of RIA equals or surpasses that of the available chromatographic and spectral methods. The added dimension of antigen–antibody specificity allows RIA's to be used for the direct analysis of compounds in physiological fluids and other biological samples that ordinarily must be processed before the other techniques can be applied. The method is nondestructive and obviates the need to use radiolabeled drugs in man during metabolic studies.

We shall describe RIA's that have been developed to quantitate nicotine and two of its metabolites and several prostaglandins and their metabolites. These assays have been used to detect the parent compound or metabolite in physiological fluids, tissue extracts, or other samples, to follow metabolic conversions, and to characterize enzyme systems. Some emphasis will be placed on examples in which HPLC has been used to separate pharmacologically active compounds and their metabolites in biological samples. The RIA can be used to quantitate the separated compounds when other detection systems are impractical, e.g., when radiolabeled derivatives cannot be used or when spectrophotometric measurements lack sensitivity and/or are complicated by the presence of endogenous components.

Nicotine and Metabolites. Recently, specific RIA's have been developed that can be used to detect picomole levels of the major tobacco alkaloid (−)-nicotine,[7] a principal metabolite, (−)-cotinine,[7] and a minor product, γ-(3-pyridyl)-γ-oxo-*N*-methylbutyramide,[13] derived from cotinine.

Nicotine and cotinine (Table I) cannot be coupled directly to carrier molecules. However, when we used succinylated 2-aminonicotine to prepare the antigenic conjugates the antiserum was not specific for nicotine, i.e., nicotine and *N*-methylpyrrolidine reacted with the antibodies to the same extent.[7] Consequently, stereospecifically synthesized carboxyl derivatives of (−)-nicotine and (−)-cotinine were covalently

(11) A. Goldstein, L. Aronow, and S. M. Kalman, "Principles of Drug Action: The Basis of Pharmacology", 2nd ed, Wiley, New York, N.Y., 1974.
(12) P. R. Brown, "High Pressure Liquid Chromatography; Biochemical and Biomedical Applications", Academic Press, New York, N.Y., 1973.
(13) J. J. Langone, J. Franke, and H. Van Vunakis, *Arch. Biochem. Biophys.*, **164**, 536 (1974).

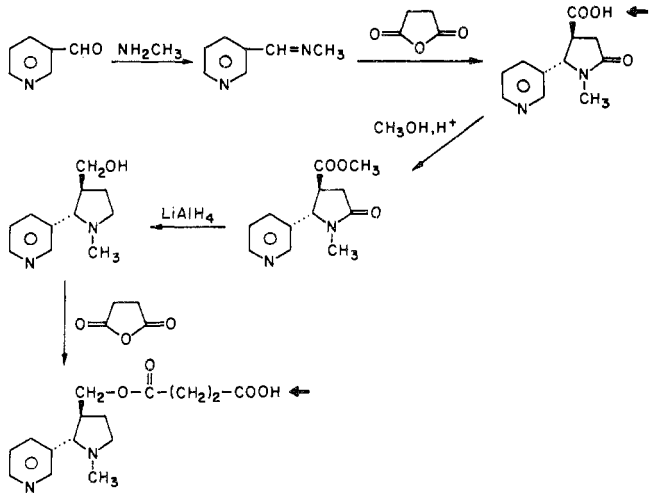


Figure 2. Preparation of hapten derivatives for conjugation to macromolecules.

linked to prepare conjugates for immunization in which both rings of each hapten were exposed in such a way that the unique structural features of each molecule were recognized by the antibody synthesizing cells (Figure 2).[7] To obtain antibodies against γ-(3-pyridyl)-γ-oxo-*N*-methylbutyramide (the oxo amide), rabbits were immunized with a conjugate prepared by coupling the corresponding carboxylic acid to amino groups of human serum albumin.[13]

[^{3}H]Nicotine was used as the labeled antigen to develop the RIA for the parent alkaloid. For the metabolites, phenolic derivatives that could be labeled with ^{125}I were prepared. *trans*-4′-Carboxycotinine and γ-(3-pyridyl)-γ-oxobutyric acid were converted to the corresponding acid chlorides and then were allowed to react *in situ* with tyramine. In addition to this procedure, we have used CDI to couple tyramine to a carboxy derivative of the insecticide dieldrin.[14a] In contrast, phenol derivatives of mescaline and 2,5-dimethoxy-4-methylphenylisopropylamine (DOM) that were suitable for iodination were prepared by coupling the amines to *p*-acetoxyphenylacetic acid chloride and then deacetylating by mild alkaline hydrolysis.[14b] These methods and the coupling of an amine (e.g., adriamycin)[14c] to *p*-hydroxyphenylacetic acid by CDI probably can be applied to other compounds that are compatible with the reagents.

The binding of the ^{125}I cotinine derivative with increments of antiserum during different courses of immunization and the inhibition of binding with known amounts of cotinine are shown in Figure 3. Similar standard curves were constructed for the antinicotine and anti-oxo amide systems. The sensitivity of the three antisera for each homologous antigen (expressed as the amount required to inhibit the Ag*–Ab reaction by 50%) and the specificities with respect to nicotine and several metabolites are shown in Table I.

With the nicotine, cotinine, and oxo amide immune systems, 50% inhibition was obtained with 0.02, 0.0067, and 0.026 nmol of homologous hapten, respectively. The lower limit for detection is 350 pg for nicotine, 200 pg for cotinine, and 400 pg for the oxo

A

Course No.	Antisera Dilution					
	1/50	1/100	1/200	1/400	1/800	1/7200
1	205	173	-	-	-	-
2	2,858	2,010	1,076	705	474	-
3	5,125	5,225	3,637	2,543	1,881	-
4	-	5,250	4,500	-	2,109	716
0	195	186	-	-	-	-

Input = 14,000 cpm

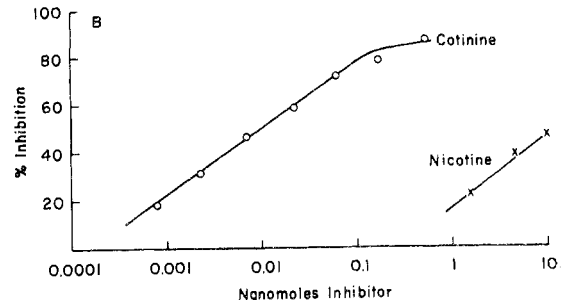


Figure 3. (A) Binding of ^{125}I-labeled cotinine derivative by rabbit antisera during different courses of immunization. Values represent counts per minute which are bound by the antibody (input = 14,000 cpm). (B) Inhibition of the ^{125}I-cotinine binding by cotinine and nicotine.[7]

amide. Although the metabolism of nicotine is complex,[15] the data in Table I indicate that the antibodies are specific and the RIA's can be used for the quantitative determination of nicotine, cotinine, and the oxo amide in the presence of each other and several other metabolites. For example, cotinine inhibits the [^{3}H]nicotine–antinicotine reaction 2500 times less effectively than nicotine, while in the anticotinine system nicotine is about 2000 times less effective than cotinine.

The geometry of the five-membered ring may be a factor in determining the specificity of these antisera. Thus antibody combining sites may recognize the "envelope" conformation of the nicotine *N*-methylpyrrolidine ring or the more planar conformation of the cotinine *N*-methyl-2-pyrrolidone ring. Furthermore, at physiological pH, nicotine would be protonated on the pyrrolidine ring. The charge contrast between the positive nicotinium ion and the neutral amide cotinine provides a major structural difference that is probably responsible in large part for the observed poor cross-reactivity.

Since the specificity of each antiserum is directed toward both rings of the homologous hapten,[7] pyridine or pyrrolidine derivatives that possess only one of the rings give essentially no inhibition at the 50-nmol level. Recently a RIA for nicotine was developed in which the immunizing conjugate was prepared by linking *N*-succinyl-6-aminonicotine to serum albumin.[16] The limited number of inhibitors that were tested, i.e., cotinine, anabasine, and nornicotine, cross-reacted poorly with the antibodies.

The RIA's have been used to analyze physiological fluids of smokers including sera, urine, and amniotic

(14) (a) J. J. Langone and H. Van Vunakis, *Res. Commun. Chem. Pathol. Pharmacol.*, **10**, 163 (1975); (b) L. J. Riceberg, H. Van Vunakis, and L. Levine, *Anal. Biochem.*, **60**, 551 (1974); (c) H. Van Vunakis, J. J. Langone, L. J. Riceberg, and L. Levine, *Cancer Res.*, **34**, 2546 (1974).

(15) H. McKennis, Jr., in "Tobacco Alkaloids and Related Compounds," U. S. von Euler, Ed., Pergamon Press, Oxford, 1965.

(16) C. F. Haines, Jr., M. S. Damodar, K. Mahajan, D. Miljkovic, M. Miljkovic, and E. S. Vesell, *Clin. Pharmacol. Ther.*, **16**, 1083 (1974).

Table II
Cotinine and Nicotine Levels in Physiological Fluids of Cigarette Smokers[7]

	M.S.[a]			K.G.[b]		
Day	ng of cotinine/ml of sera	24 hr urine mg of cotinine	24 hr urine ng of nicotine	ng of cotinine/ml of sera	24-hr urine mg of cotinine	24-hr urine mg of nicotine
3[c]	134	2.2	0.7	200	5.1	1.0
2[c]	172	3.0	1.1	170	6.1	1.2
1[c]	170	2.4	0.3	260	5.2	2.7
1[d]	150	2.8	0.2	170	3.2	0.42
2[d]	60	1.6	0.1	75	2.4	0.28
3[d]	38	1.0	<0.1	30	1.6	0.6
4[d]	12	0.2		20	0.8	0.6
5[d]	5			15		
8[d]	<2	<0.1	<0.1	<2	<0.1	<0.1

[a] M.S. is a 53-year old male who has smoked 1–2 packs of cigarettes per day for 28 years. [b] K.G. is a 23-year old male who has smoked 1 pack of cigarettes per day for 4 years. [c] Days *before* subject stopped smoking. [d] Days *after* subject stopped smoking.

and spinal fluids.[7,13,16,17] Other methods, including TLC,[18] GLC,[19] and mass spectroscopy,[20] also have been used for similar purposes. In smokers' sera the major metabolite, cotinine, is found in concentrations ranging up to 650 ng/ml. The nicotine content usually does not exceed 50 ng/ml. While the half-life of nicotine is less than 30 min,[19] that of cotinine is approximately 20–30 hr.[7,17a] The data in Table II show the levels of nicotine and cotinine in the urine and levels of cotinine in the sera of two typical smokers before and after they stopped smoking. Once an individual refrained from smoking, several days were required before the alkaloid and the metabolite finally were eliminated from blood and urine.

Probably due to differences in smoking habits and/or other physiological factors yet to be described, the correlation between cotinine levels in blood and the nicotine contained in the cigarettes an individual claims to smoke daily is poor.[17a] Since there is a lack of marked variation in the serum cotinine levels of individuals who smoke according to relatively consistent routine patterns, it may be advantageous to assay for cotinine in epidemiological studies designed to relate the effects of smoking to health.

The number of subjects in our studies has been relatively small, and the state of their health has not been monitored long enough to allow significant conclusions to be drawn regarding the relation of nicotine and cotinine levels to the physiological effects of smoking. However, the feasibility of using these RIA's to determine simply and accurately the levels of nicotine and cotinine in the physiological fluids of large numbers of people has been demonstrated. For epidemiological studies, for example, the cotinine RIA can be carried out on peripheral blood collected from the finger.

As an indication of the sensitivity of the RIA's it was possible to detect nicotine and cotinine in the sera of a subject who had not smoked for 2 weeks and then smoked consecutively two cigarettes of known nicotine content (2.13 mg/cigarette). The values for the prebleeding and for the samples collected 5 min, 24 hr, and 48 hr after smoking were: <0.2, 38, 29, and 7 ng of cotinine/ml of sera, respectively; and <0.5, 38, <0.5, and <0.5 ng of nicotine/ml of sera for the same time periods.[17a] In cases where the levels of cotinine and nicotine are low, it was necessary to extract the sera to concentrate these compounds prior to analysis.

Since the symptoms of green tobacco sickness, i.e., dizziness, pallor, prostration, and nausea, resemble the symptoms observed in novice smokers, nicotine absorbed by tobacco harvesters through dermal contact with the wet tobacco leaves has been suspected as the likely cause of the disease.[21a] Analysis by RIA showed that there was a 16-fold rise in mean excretion of cotinine among workers who had greatest contact with the tobacco and who were most susceptible to the disease.[21b] (Cotinine occurs naturally in tobacco only in trace amounts, and exogenous contamination of samples with this metabolite is unlikely). Less cotinine was found in urine of workers who had less exposure. Although the possibility exists that nicotine is only a marker for another poison that causes the disease, dermal absorption of the potent alkaloid is most likely the etiology for tobacco sickness.

The in vitro oxidation of nicotine to cotinine and of cotinine to the oxo amide by oxidases present in rabbit liver also could be followed by RIA.[7,13] HPLC proved to be a useful independent quantitative method to check the RIA results when [^{3}H]nicotine or [^{3}H]cotinine was used in the incubation. The correlation between the two methods is good for both enzymatic reactions (Table III). The effect on activity of soluble and microsomal fractions, cofactors, and variables such as pH and ionic strength could be determined. The K_M and K_i values were obtained by analyzing the incubation mixtures directly by RIA.

Prostaglandins and Metabolites. Prostaglandins were discovered in the mid-thirties by von Euler,[22] but extensive investigations of their chemical and bi-

(17) (a) J. J. Langone, H. Van Vunakis, and P. Hill, *Res. Commun. Chem. Pathol. Pharmacol.*, **10**, 21 (1975); (b) H. Van Vunakis, J. J. Langone, and A. Milunsky, *Am. J. Obstet. Gynecol.*, **120**, 64 (1974).

(18) D. M. Turner, *Biochem. J.*, **115**, 889, (1969).

(19) (a) I. E. Burrows, P. J. Carp, G. C. Jackson, and B. F. Page, *Analyst*, **96**, 81 (1971); (b) A. H. Beckett, J. W. Gorrod, and P. Jenner, *J. Pharm. Pharmacol.*, **23**, Suppl. 55S (1971); (c) M. A. H. Russell and C. Feyerbend, *Lancet*, 179 (1975); (d) P. F. Isaac and M. J. Rand, *Nature (London)*, **236**, 308 (1972).

(20) E. C. Horning, M. G. Horning, D. I. Carroll, R. N. Stillwell, and I. Dzidic, *Life Sci.*, **13**, 1331 (1973).

(21) (a) S. H. Gehlbach, W. A. Williams, L. D. Perry, and J. S. Woodall, *J. Am. Med. Assoc.*, **229**, 1880 (1974); (b) S. H. Gehlbach, L. D. Perry, W. A. Williams, J. I. Freeman, J. J. Langone, L. V. Peta, and H. Van Vunakis, *Lancet*, 478 (1975).

Table III
Enzymatic Oxidation by Rabbit Liver Extracts of (−)-Nicotine to (−)-Cotinine (Experiment 1) and of (−)-Cotinine to γ-(3-Pyridyl)-γ-oxo-*N*-methylbutyramide (Experiment 2)[a]

Time, min	% conversion determined by Radioimmunoassay	% conversion determined by Liquid chromatography
	Experiment 1	
0	0.13	0.19[b]
20	5.90	6.90
40	9.97	11.8
60	15.2	18.0
80	17.2	19.2
	Experiment 2	
0	0.15	0.35[c]
120	3.2	3.6
240	4.8	5.3
360	6.0	6.2

[a] Each reaction mixture contains liver extract, ^3H substrate, and NADPH. Other experimental details are given in ref 7 and 13. [b] Cpm in cotinine peak. [c] Cpm in oxo amide peak.

ological properties were begun only about a decade ago. Since then the structures of prostaglandins have been elucidated by Bergstrom and collaborators,[23] and the chemical synthesis of several prostaglandins has been achieved by Axen et al.[24] and Corey et al.[25]

Prostaglandins are a family of C_{20} unsaturated fatty acids that contain a cyclopentane ring and two aliphatic side chains. They are divided into different types by structural differences on the cyclopentane ring. While prostaglandins of the F type have two hydroxyl groups on the cyclopentane ring, prostaglandins of the E type contain one hydroxyl and one keto group (Figure 4). PGA, PGC, and PGB are isomers having, in addition to one keto group, one double bond at different positions in the ring. The number of double bonds in the aliphatic side chain divides prostaglandins of each type into three classes: PGE_1, PGE_2, and PGE_3 have one, two, and three double bonds, respectively, in the two side chains. Only PGF and PGB are stable to elevated temperature and over a wide pH range. PGE is converted to PGA at acid pH. PGE, PGA, and PGC are converted to PGB under alkaline conditions.[26a] PGC is especially sensitive to acid and alkali[26b] and is converted slowly to PGB even at neutral pH.[26c]

The physiological role of prostaglandins has been studied[27] extensively, and many diverse actions of prostaglandins have been reported. While prostaglandins of class two (PGE_2, PGF_2, etc) are found in higher concentrations and are usually more potent than those of class one or three, the activity of one type (PGE_1, PGE_2, PGE_3) is generally, at least qualitatively, the same regardless of the degree of unsaturation. Also the known prostaglandin enzymes are thought to act on prostaglandins of different degrees of unsaturation in the same way.

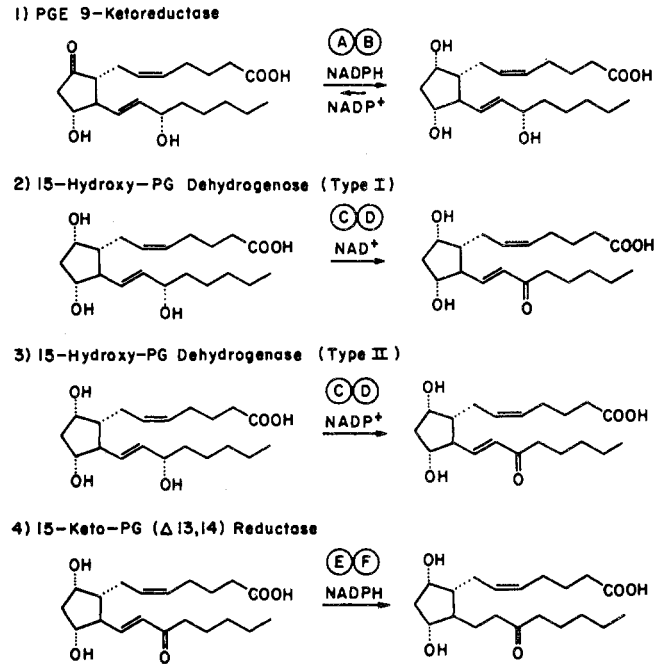


Figure 4. Enzymatic conversions of some prostaglandins: (1) reduction of C-9 keto group; (2 and 3) oxidation of C-15 hydroxyl group; (4) reduction of C-13,14 double bond.

An important area of study has been the development of analytical procedures for the quantitative determination of the prostaglandins in tissues and biological fluids. Chemical analysis is complex and time consuming. It involves extraction, one or two chromatographic separations, and gas chromatographic and mass spectroscopic analysis for chemical identification. Since Levine and Van Vunakis first reported production of antibodies to prostaglandins in 1970,[28] antibodies have been prepared to many prostaglandins and several metabolites.[29] As in all immune procedures for antigen determination, the serologic specificities of the antigen–antibody reactions determine the usefulness of the antibodies. The RIA's for prostaglandins are now being used widely to elucidate the physiological role of prostaglandins and have been used to detect enzymes that regulate and/or metabolize prostaglandins.

In Figure 4 are shown three enzymatic reactions: (1) enzymatic reduction of the C-9 keto group to an hydroxyl group (conversion of PGE_2 to $PGF_{2\alpha}$); (2 and 3) enzymatic dehydrogenation of the C-15 hydroxyl to a keto, leading to loss of pharmacological activity of the prostaglandin;[30] and (4) enzymatic reduction of the C-13,14 double bond.

The serologic specificities relevant to the assay for

(22) U. S. von Euler, *J. Physiol.*, **88**, 213 (1936).
(23) S. Bergstrom, R. Ryhage, B. Samuelsson, and J. Sjevall, *Acta Chim. Scand.*, **16**, 501 (1962); *J. Biol. Chem.*, **238**, 3555 (1962).
(24) U. Axen, J. L. Thompson, and J. E. Pike, *Chem. Commun.*, 602 (1970).
(25) E. J. Corey, S. M. Albonico, N. Koelliker, T. K. Schaaf, and R. K. Varma, *J. Am. Chem. Soc.*, **93**, 1491 (1971).
(26) (a) N. H. Anderson, *J. Lipid Res.*, **10**, 329 (1969); (b) E. J. Corey and G. Moinet, *J. Am. Chem. Soc.*, **95**, 1785 (1973); (c) R. L. Jones, *J. Lipid Res.*, **10**, 511 (1972).
(27) E. W. Horton, *Proc. R. Soc. London, Ser. B*, **182**, 411 (1972).
(28) (a) L. Levine and H. Van Vunakis, *Biochem. Biophys. Res. Commun.*, **41**, 1171 (1970); (b) L. Levine, R. M. Gutierrez-Cernosek, and H. Van Vunakis, *J. Biol. Chem.*, **246**, 6782 (1971).
(29) (a) B. M. Jaffe, J. W. Smith, W. T. Newton, and C. W. Parker, *Science*, **171**, 494 (1971); (b) K. T. Kirton, J. C. Cornette, and K. L. Barr, *Biochem. Biophys. Res. Commun.*, **47**, 903 (1972); (c) R. M. Zusman, B. V. Caldwell, L. Speroff, and H. R. Behrman, *Prostaglandins*, **2**, 41 (1972); (d) S. C. Yu and G. Burk, *ibid.*, **2**, 11 (1972); (e) B. V. Caldwell, S. Burstein, W. A. Brock, and L. Speroff, *J. Clin. Endocrinol. Metab.*, **33**, 171 (1971); (f) E. Granstrom and B. Samuelsson, *FEBS Lett.*, **26**, 211 (1972); (g) S. Bauminger, U. Zor, and H. R. Lindner, *Prostaglandins*, **4**, 313 (1973).
(30) B. Samuelsson, *Fed. Proc., Fed. Am. Soc. Exp. Biol.*, **31**, 1442 (1972).

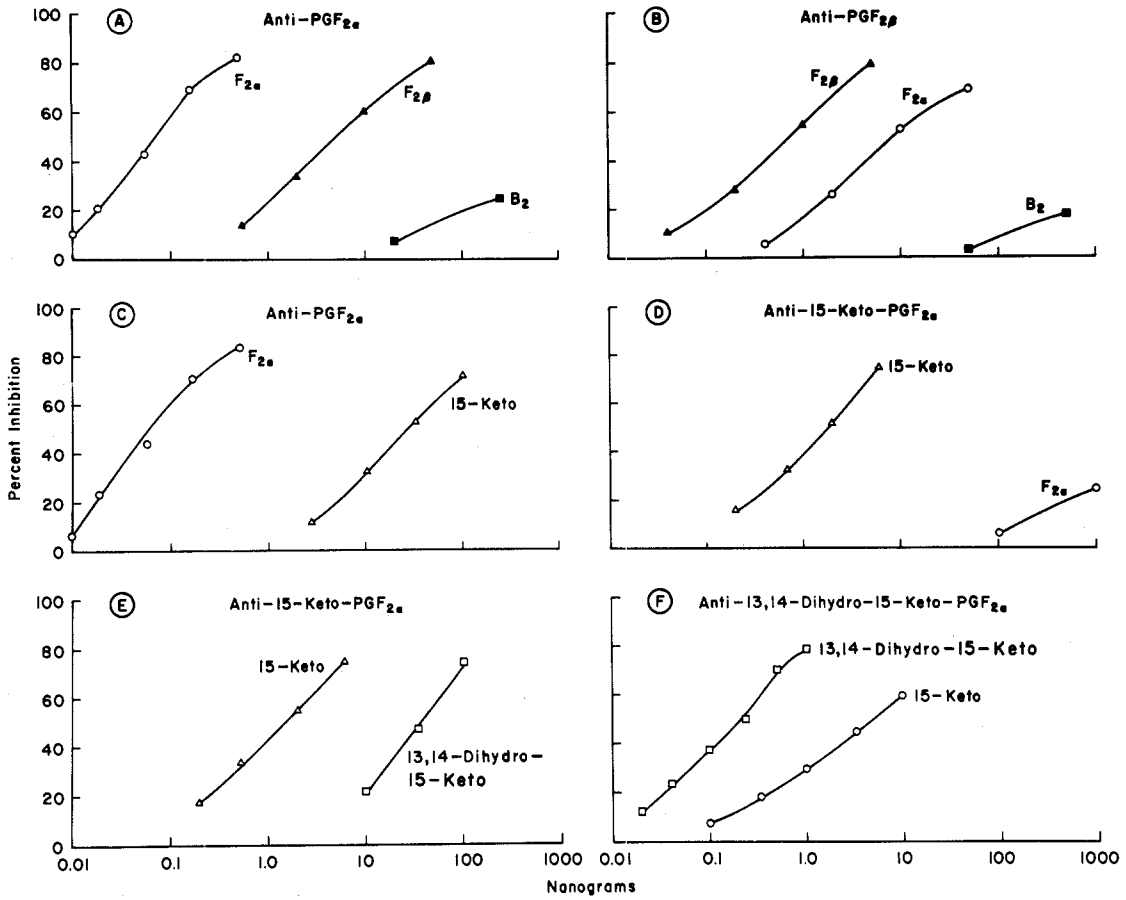


Figure 5. Serological specificities of some prostaglandin antisera.

each enzymatic reaction are shown in Figure 5. For enzymatic reactions 2, 3, and 4, RIA's were developed for both the substrate and the product so that catalysis could be measured in two ways: loss of substrate and concomitant generation of product. For example, to follow the dehydrogenation of $PGF_{2\alpha}$, antibodies to the substrate $PGF_{2\alpha}$ (C) that react only about 1% as effectively with the product and antibodies to the product (15-keto-$PGF_{2\alpha}$) (D) that react less than 1% as effectively with the substrate are used.[31] To detect enzymatic reduction of the 13,14 double bond, antibodies to the substrate (15-keto-$PGF_{2\alpha}$) (E) that react with the product only about 5% and antibodies to the product (13,14-dihydro-15-keto-$PGF_{2\alpha}$) (F) that react with the substrate about 10% are used.

Enzymatic conversion of PGE to PGF (reaction 1) can be followed by RIA with antibodies to the substrate "PGE_2" and to the product $PGF_{2\alpha}$ but only the specificities of the antisera to the potential products $PGF_{2\alpha}$ (A) and $PGF_{2\beta}$ (B) are shown in Figure 5. The antiserum to the substrate PGE_2 is directed toward its alkali-labile product (PGB_2), so that to measure enzymatic activity residual substrate is determined as PGB_2 after dehydration with NaOH ($PGF_{2\alpha}$ is stable to this treatment).[32] The product of enzymatic reduction ($PGF_{2\alpha}$) reacts less than 0.1% with antibodies to this PGB_2. As can be seen in Figure 5, (A and B), the antisera to the potential products of enzymatic reduction of the 9-keto group of PGE_2, do not react effectively with the NaOH-treated substrates (PGB_2).

(31) L. Levine, *Pharmacol. Rev.*, **25**, 293 (1973).
(32) S. C. Lee and L. Levine, *J. Biol. Chem.*, **249**, 1369 (1974).

The specificities of the antibodies of $PGF_{2\alpha}$ and $PGF_{2\beta}$ permit determination of the stereoselectivity of the enzymatic reduction. Based on the use of both antisera to measure levels of PGF_2, at least 90% of the product is $PGF_{2\alpha}$.

With the use of these RIA procedures,[33] the distribution of two enzymes which catalyze the reduction of the 9-keto group of PGE to form PGF has been determined in several tissues from different species. These enzymes differ in their cofactor requirements and physical properties. The PGE 9-ketoreductases can reduce the 9-keto group and 15-keto group of 15-keto-PGE_2 and the 15-keto group of 15-keto-$PGF_{2\alpha}$ to the corresponding hydroxyl group. PGD_2, a PG with a 9-hydroxyl and an 11-keto group, is not converted to $PGF_{2\alpha}$, nor is cyclohexanone reduced to cyclohexanol by PGE 9-ketoreductase.

When homogenates of several mammalian tissues were analyzed by RIA for 15-hydroxyprostaglandin dehydrogenase activity, two types of enzyme activity were detected. Both types were partially purified, one from monkey brain and the other from chicken heart. In addition to different cofactor requirements, the two partially purified enzymes were distinguished by chromatographic properties, their relative affinities for PGE_2 and $F_{2\alpha}$, and their sensitivities to inhibition by reduced pyridine nucleotides, thyroid hormones, and PGB_2.

(33) (a) S. C. Lee and L. Levine, *J. Biol. Chem.*, **250**, 548 (1975); (b) S. C. Lee and L. Levine, *ibid.*, **250**, 4549 (1975); (c) S. C. Lee, S. S. Pong, D. Katzen, K. Y. Wu, and L. Levine, *Biochemistry*, **14**, 142 (1975); (d) L. Kaplan, S. C. Lee, and L. Levine, *Arch. Biochem. Biophys.*, **167**, 287 (1975); (e) S. C. Lee and L. Levine, *Biochem. Biophys. Res. Commun.*, **61**, 14 (1974).

Conclusion

This Account describes how antibodies specific for nicotine and its metabolites and for various prostaglandins and their metabolites have been used to develop sensitive RIA's for these compounds. Thus far, antibodies have been prepared and RIA's developed for at least 100 other compounds of pharmacological importance.[1,3-6] It has been calculated that an individual animal has the potential to produce antibodies that can recognize 10^7 and perhaps up to 10^8 diverse immunodominant moieties. Nature, therefore, has provided the pharmacologist and synthetic chemist with an analytical system of extraordinary specificity that can be adapted to the quantitative determination of a wide variety of pharmacologically active molecules. It is clear that RIA will be an important analytical tool in research as well as in the clinical laboratory.

Some of the work described in this account was supported by Research Contract NCI E-72-3243 from the National Cancer Institute (H.V.V.), Grant No. DA 00007 from the National Institute on Drug Abuse (H.V.V.), Grant HD-07966 from the National Institute of Child Health and Development (L.L.), and Grant IM-22M from the American Cancer Society (L.L.). H.V.V. holds a Research Career Award (Award KO6-AIO2372) from the National Institute of Allergy and Infectious Diseases. L.L. is an American Cancer Society Professor (Award No. PRP-21).

Disodium Tetracarbonylferrate—a Transition-Metal Analog of a Grignard Reagent

James P. Collman

Department of Chemistry, Stanford University, Stanford, California 94305

Received February 19, 1975

With the expectation that significant synthetic methodologies will emerge from organotransition-metal chemistry, organic chemists are becoming increasingly interested in this rapidly growing field. To date, few truly useful new organotransition reagents have been developed.[1] However as new reactions are discovered and the underlying reaction mechanisms are slowly clarified, practical applications become more likely. In this Account, I summarize our research on $Na_2Fe(CO)_4$ as a reagent for organic synthesis.

This work had its origin in 1970 when my postdoctoral associate, M. Cooke, was searching for a method of forming Ge–Ge bonds by coupling R_3GeCl with two electron reductants such as $Na_2Fe(CO)_4$. Being an organic chemist, Cooke tried methyl iodide in a model reaction. Treatment of CH_3I with $Na_2Fe(CO)_4$ followed by hydrolysis gave the characteristic odor of acetaldehyde. This lead was quickly developed into a general synthesis of homologous aldehydes.[2] Because of my past interest in oxidative addition,[3] reductive elimination,[3b] and migratory insertion,[3b] the potential of $Na_2Fe(CO)_4$ as a reagent for organic synthesis was evident, and the matter became vigorously pursued by my other students.

Synthesis of the Reagent. Our early experiments employed $Na_2Fe(CO)_4$ derived from $Fe(CO)_5$ and sodium–mercury amalgam (eq 1).[2] Because of the expense, difficulty in scale-up, and the presence of mercury salts and of colored polynuclear iron carbonyl impurities inherent in this procedure, we sought a better method for preparing $Na_2Fe(CO)_4$. Eventually we developed a very practical method[4,5] (eq 2)

$$\underset{\text{orange}}{Fe(CO)_5} \xrightarrow[THF]{Na(Hg)} \underset{\text{red-yellow}}{Na_2Fe(CO)_4} \quad (1)$$

$$\underset{\text{orange}}{Fe(CO)_5} + Na \xrightarrow[C_6H_5COC_6H_5]{\text{dioxane, } 100^\circ} \underset{\text{deep blue}}{\text{[}Na_2Fe(CO)_4\text{]}} \cdot 1.5\text{dioxane} \quad \underset{\text{white}}{} \quad (2)$$

employing $Fe(CO)_5$, the least expensive iron carbonyl,[6] and metallic sodium, with an electron carrier (such as benzophenone ketyl) in an ethereal solvent under conditions where the sodium (mp 97.5°) is molten. At atmospheric pressure, boiling dioxane (bp 101°) is ideal, yielding a more soluble solvate (eq 2). This process is rapid, nearly quantitative, and easily scaled up. Present raw material costs in this preparation of $Na_2Fe(CO)_4$ depend substantially (~75%) on the current price of $Fe(CO)_5$. However, if a large-scale application for $Fe(CO)_5$ were developed,[7] raw material costs could drop below those of Grignard re-

Professor Collman's work covers a wide range of complex ion chemistry, from the classical coordination compounds to the newer organometallic combinations. He is a native of Nebraska, and studied at the University of Nebraska for his B.S. degree. Following receipt of the Ph.D. from the University of Illinois in 1958, he joined the faculty of University of North Carolina. In 1967, he moved to Stanford University, where he is professor of chemistry.

(1) Perhaps the most versatile and useful transition-metal reagents developed thus far are organocopper compounds: H. O. House, *Proc. Robert A. Welch Found. Conf. Chem. Res.*, **17**, 101 (1973).

(2) M. P. Cooke, *J. Am. Chem. Soc.*, **92**, 6080 (1970).

(3) (a) J. P. Collman and W. R. Roper, *J. Am. Chem. Soc.*, **87**, 4008 (1965); (b) J. P. Collman, *Acc. Chem. Res.*, **1**, 136 (1968); (c) J. P. Collman and W. R. Roper, *Adv. Organometal. Chem.*, **7**, 54 (1968).

(4) J. P. Collman and R. G. Komoto, U.S. Patent Application filed June 11, 1973.

(5) (a) J. P. Collman, R. G. Komoto, W. O. Siegl, S. R. Winter, and D. R. Clark, unpublished results; (b) S. R. Winter, Ph.D. Dissertation, Stanford University, 1973; (c) R. G. Komoto, Ph.D. Dissertation, Stanford University, 1974.

(6) In fact, $Fe(CO)_5$ is the least toxic and least expensive transition-metal carbonyl. The other iron carbonyls, $Fe_2(CO)_9$ and $Fe_3(CO)_{12}$, are derived from $Fe(CO)_5$ and thus are more expensive.

(7) For example, commercialization of a carbon monoxide process converting ilmenite to rutile would produce $Fe(CO)_5$ as a by-product: A. Vasnapu, B. C. Marek, and J. W. Jensen, Report of Investigations 7719, U.S. Department of the Interior, Bureau of the Mines, 1973.