RECENT DEVELOPMENTS IN CHEMICAL AND BIOCHEMICAL ASSAY TECHNIQUES APPLICABLE IN PHARMACOLOGY[1,2]

By R. P. Maickel and H. Weissbach

Laboratory of Chemical Pharmacology and Laboratory of Clinical Biochemistry, National Heart Institute, National Institutes of Health, Bethesda, Maryland

Although pharmacological research utilizes many different assay techniques to determine substances in biological systems, a void exists in the pharmacological literature in the area of analytical techniques. The majority of reviews on physical, chemical, and biochemical methods of assay for substances of interest to the pharmacologist are found in chemical journals and often are too specialized to be of value. The textbooks of pharmacology and allied disciplines contain only brief discussions of assay methods, seriously limiting their value in this field.

This review describes three relatively new analytical techniques useful in pharmacological research: spectrophotofluorometry, gas-liquid chromatography, and the combined technique of tritium-gas exposure labeling and liquid scintillation counting. Applications rather than theory are emphasized, so that the reader may develop a feel for using the techniques. Wherever possible, we have discussed the application of these techniques to problems of interest to the pharmacologist. The scope of this review is, of course, somewhat limited, but it is the hope of the authors that other reviews of this type will be made.

Spectrophotofluorometry

Although fluorescent assay procedures have been available to the pharmacologist and biochemist for many years, the advent of new instrumentation has brought this technique into more prominence in the past five years. Conventional filter fluorometers could only be used for compounds which absorbed light at wavelengths coinciding with one of the lines of the mercury emission spectrum and emitted light of visible wavelengths. The recent development of the spectrophotofluorometer (1) extended the scope of fluorescence analysis by permitting both the excitation of compounds and the measurement of the resulting fluorescence throughout the ultraviolet and visible regions. The basic components of the spectrophotofluorometer are a high-intensity xenon arc source which emits continuously throughout most of the ultraviolet and visible regions and two diffraction-grating monochromators, one to separate monochromatic light for excitation and a second

[1] The survey of the literature pertaining to this review was concluded in July 1961.

[2] Abbreviations used in this chapter include: GLC (gas-liquid chromatography).

monochromator at right angles to the first to analyze the emitted fluorescence. A photomultiplier circuit measures the intensity of the fluorescence.

The advantages of this type of instrumentation are numerous. The diffractiongratings permit accurate comparison of different fluorescent samples, since recording instruments can be readily attached to the spectrophotofluorometer. Graphical results of fluorescence intensity versus the wavelength of activating or emitting light are obtained in this manner. The fluorescent peaks corresponding to activation and emission maxima are often of value in indentifying an unknown compound, since similar basic structures often have similar fluorescence characteristics. However, it must be remembered that fluorescent wavelengths may vary from instrument to instrument, and a correction factor is often needed.

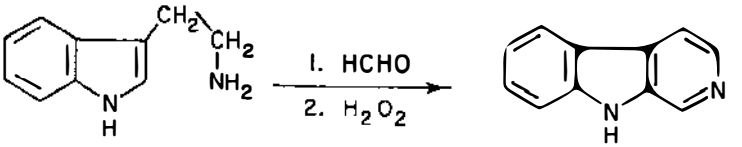
A series of compounds of pharmacologic (2) and biochemical interest (3), many not previously shown to fluoresce, have native fluorescence when activated by ultraviolet light. A large number of assays have been published based on the initial findings with the spectrophotofluorometer.

Fluorometric techniques have been especially useful in the assay of biogenic amines, many of which are present in tissues in only minute amounts. Formerly, only bioassay procedures possessed the necessary sensitivity. Sensitive fluorometric procedures have now been published for the assay of many pharmacologically active amines, their metabolites, and the enzymes involved in their biosynthesis and metabolism. We have selected this area as a particularly appropriate field for a review of spectrophotofluorometric techniques.

Indoleamines and related compounds.—The intense fluorescence of the indole nucleus has been used in assay methods for tryptophan and its metabolites. For example, of the amino acids present in protein hydrolysates, only trytophan and tyrosine fluoresce. At pH 11, the fluorescence maxima of tryptophan is 350 mμ, that of tyrosine is 310 mμ. Since the fluorescence of tryptophan is 100 times greater than that of tyrosine, tryptophan may be determined in the presence of an excess of tyrosine without prior separation (4). Tryptophan may also be measured in plasma after removal of proteins with tungstic acid (4).

The determination of tryptamine in biological material has become important because of recent work on monoamine oxidase inhibitors. Sjoerdsma *et al.* (5) have described an assay for tryptamine in urine. The amine is extracted into benzene, re-extracted into dilute HCl, and assayed by its native fluorescence in alkaline solution. Urinary tryptamine levels are a sensitive index of monoamine oxidase inhibition *in vivo*. To determine the minute amounts of tryptamine present in tissues, Hess & Udenfriend (6) developed a sensitive assay based on the oxidation of tryptamine to norharman. The amine is isolated by solvent extraction, then converted by condensation and oxidation to a highly fluorescent norharman derivative. This procedure has been used to measure the increased tryptamine levels in tissues after administration of iproniazid and tryptophan. Tryptophan may also be converted to

a fluorescent norharman derivative which may permit the determination of tryptophan in small quantities of tissue. Tryptamine reacts with ninhydrin on paper chromatograms (7) to yield highly fluorescent derivatives which can be assayed in eluates (8).



TRYPTAMINE NORHARMAN

The assay of serotonin was perhaps the first reported use of the spectrophotofluorometer for quantitative determinations (9). The fluorescent characteristics of serotonin and other 5-hydroxyindoles have been described (10). In strong acid solution (3N HCl) the maximum fluorescence peak appears at 540 mμ. This fluorescence in strong acid is quite specific for 5-hydroxyindoles. The extraction method of Bogdanski *et. al.* (11) is commonly employed for assay of serotonin in brain. Methods also exist for determination of serotonin in platelets (9, 12) and in human whole blood (13). Since serotonin is the only 5-hydroxyindole normally found in many tissues, it is possible to determine this amine directly after protein precipitation (14). This procedure is adequate where tissue levels are above 1 μg per g, but an extraction procedure must be employed in tissues, such as brain, where the serotonin content is lower, or when other 5-hydroxyindoles may be present. Oates (15) has described the determination of serotonin in urine of malignant carcinoid patients after isolating the amine by ion exchange chromatography. Urine is an especially difficult sample due to low serotonin content and many interfering substances.

5-Hydroxytryptophan is not normally present in significant amounts in tissues, but large amounts (> 5 μg/g tissue) are found after administration of the amino acid to animals (16). Since some patients with malignant carcinoid have been shown to excrete this amino acid in their urine (17), Sjoerdsma *et al.* (18) have described a method in which 5-hydroxytryptophan is isolated from urine, enzymatically converted to serotonin (19), and fluorometrically determined as the latter amine.

The major metabolite of serotonin is 5-hydroxyindoleacetic acid, usually assayed by colorimetry after reaction with 1-nitroso-2-naphthol (20). The microgram quantities of 5-hydroxyindoleacetic acid present in tissues after administration of either 5-hydroxytryptophan or serotonin may be determined by a fluorometric procedure (16). The acid is isolated by ether extraction of the acidified tissue extract, then re-extracted into buffer pH 7.0.

Lerner and co-workers (21, 22) have identified melatonin as 5-methoxy-*N*-acetyltryptamine. The trace quantities of this hormone normally found

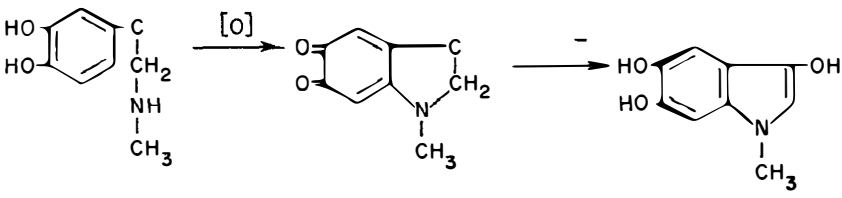
in the pineal gland required the use of a bioassay, based on the ability of the indole to lighten frog skin. However, in enzyme studies with hydroxyindole-*O*-methyltransferase (23), a simple extraction procedure has been used to assay microgram quantities of melatonin.

The methods employed for the assay of 5-hydroxyindole compounds and related compounds have been reviewed by Udenfriend *et al.* (24).

Tyramine, the catechol amines and related compounds.—The reaction of tyrosine with 1-nitroso-2-naphthol is the basis for a simple colorimetric assay for tyrosine (25). Waalkes & Udenfriend (26) have developed a sensitive method for the determination of tyrosine in plasma and tissues by utilizing the fluorescence of the nitroso-naphthol derivative. Chirigos *et al.* (27) and Guroff & Udenfriend (28) have studied tyrosine uptake by mammalian tissues using this method.

Nitroso-naphthol also reacts with tyramine and has been used to identify this amine in fruits and vegetables (29). After extraction into ether from tissue homogenate at pH 10, the tyramine is re-extracted into dilute acid and coupled with nitroso-naphthol. p-Hydroxyphenylacetic acid, a major metabolite of tyramine, is found in normal urine (30). The fluorescence of the acid is determined at 310 mμ after ether extraction; the method is similar to that used for other aromatic acids such as salicylic acid (31). This procedure has been used to study transport of p-hydroxyphenylacetic acid into tissues (27, 28, 32), and a similar extraction procedure has been employed for assay of 3,4-dihydroxymandelic acid in enzymatic experiments (33).

Catechol amines and related compounds.—Many assay procedures for the catechol amines, i.e., 3,4-dihydroxyphenylethylamine (dopamine), norepinephrine and epinephrine, have been reported, although in most instances only modifications of a basic method are presented. The initial problem is isolation of the catechol amine from tissue. The most commonly used procedure is adsorption on alumina (34, 35), although ion exchange resins have also been employed (36), and an extraction procedure has been described (37). Once isolated, catechol amines can be oxidized to pink aminochromes by manganese dioxide (38), ferricyanide (39, 40), or iodine (41). The addition of alkali converts the aminochromes to highly fluorescent di- or trihydroxyindole derivatives (35, 39).



EPINEPHRINE ADRENOCHROME 1-METHYL-3,5,6 TRIHYDROXYINDOLE

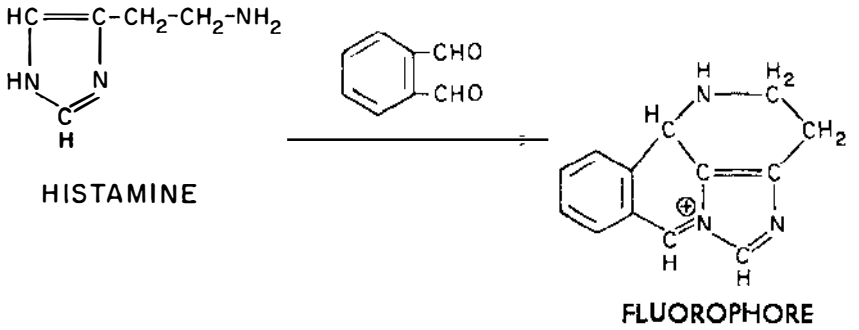
The method of Carlsson & Waldeck (42) for the assay of dopamine is based on the formation of a dihydroxyindole fluorophore, under conditions in which the corresponding fluorophores of norepinephrine and epinephrine do not interfere. The amine fluoresces at 410 mμ after oxidation, rearrangement, and irradiation with ultraviolet light. The method has been applied to tissues after isolation of dopamine by means of a cation exchange resin (Dowex 50 H) (36).

The assay of norepinephrine and epinephrine in urine has been discussed in great detail by Crout (43). The most satisfactory procedure involves the use of alumina to isolate catechol amines, followed by conversion to trihydroxyindole derivatives. By performing the final oxidation in duplicate samples at pH 6.5 and 3.5, it is possible to differentiate between norepinephrine and epinephrine since the former compound oxidizes very slowly at the lower pH.

Shore & Olin (37) employed an extraction procedure to isolate norepinephrine from brain and other tissues. The solubility of norepinephrine hydrochloride permits the extraction of the catechol amine from acidified tissue into butanol. Addition of heptane to the butanol lowers the solubility of the catechol amine in the butanol phase and permits re extraction into dilute acid for assay by a modification of the method of Lund (35), using thiosulfate to destroy the excess oxidant (44). This butanol extraction procedure also isolates serotonin from tissues, since the method is similar to that described by Udenfriend *et al.* (9). Mead & Finger (45) have assayed both norepinephrine and serotonin in the same tissue extract by extraction of the amines as described by Shore & Olin (37), followed by fluorometric assay of different aliquots of the final acid extract. This procedure has been modified by Kuntzman *et al.* (46) to permit the determination of as little as 40 m μg of norepinephrine and 200 m μg of serotonin in nervous tissue.

Since a large percentage of circulating catechol amines are metabolized by *O*-methylation, several assays for metanephrine derivatives have been developed. Bertler *et al.* (47) have described a method based on oxidation of the metanephrines and rearrangement of the oxidized products to trihydroxyindole derivatives. Smith & Weil-Malherbe (48) have described the differential estimation of metanephrine and normetanephrine in urine. The urine is hydrolyzed and the catechols are removed by passage over alumina. The metanephrines are isolated by adsorption on Amberlite CG-50 columns, eluted, and oxidized to fluorescent indole derivatives.

Histamine.—A fluorometric procedure for the assay of histamine has been developed by Shore *et al.* (49). The method has the sensitivity and specificity necessary to measure histamine in tissues, and represents an important advance in a field where an adequate assay was lacking. The procedure involves the reaction of histamine with *O*-phlthalaldehyde in alkaline solution to produce a sensitive acid-stabilized fluorophore (excitation 360 mμ, fluorescence 450 mμ). The histamine is extracted from deproteinized tissue extracts into butanol, washed with dilute alkali, and re-extracted into acid for the



condensation step. Of a number of related compounds including acetylhistamine, imidazoleacetic acid, methylhistamine, carnosine, and N-alkylhistamines, only ammonia (at concentrations >4 μg/ml) was found to interfere. Histidine reacts with *o*-phthalaldehyde but is removed by the alkali wash. Since as little as 0.02 μg of histamine can be detected, this method may replace bioassay procedures for the measurement of normal tissue histamine levels.

Fluorometric assay of enzymes involved in amine metabolism.—The enzyme, aromatic L-amino acid decarboxylase is found in mammalian tissues such as kidney, liver, and brain. This enzyme is responsible for the decarboxylation of a variety of aromatic amino acids such as 5-hydroxytryptophan, 3,4-dihydroxyphenylalanine, tryptophane, tyrosine, and phenylalanine (50). With the exception of phenylalanine, all these decarboxylations may be studied by fluorometric determination of the products. The amine products can be separated on ion exchange resins (51). In the procedure described by Dietrich (52), the enzymatic activity is destroyed by heating, and an aliquot of the incubation mixture is passed over a small permutit column. After washing the column with water to remove the amino acid, the amine is eluted with a salt solution. Several amines, including serotonin, dopamine, tyramine, and tryptamine, can be assayed by their native fluorescnece directly in the saline eluates.

Fluorescent procedures have been employed for the assay of monoamine oxidase in small amounts of tissue, or in tissues with low enzyme activity. Serotonin (53) or tryptamine (54) can be used as the substrate, and if the reaction mixture contains saturating levels of both aldehyde dehydrogenase and nicotinamide-adenine dinucleotide (NAD),[3] the amines are converted stoichiometrically to the corresponding acids (5-hydroxyindoleacetic acid or indoleacetic acid) which can be assayed by fluorometric procedures.

[3] In accordance with the newly recommended usage [see *Nature*, **188**, 464–66 (November 5, 1960); *Science*, **132**, 1548–60 (November 25, 1960)], nicotinamide-adenine dinucleotide (NAD) has been substituted for diphosphopyridine nucleotide (DPN).—EDITOR.

Diamine oxidase has been assayed by measuring histamine or agmatine disappearance (55) with greater sensitivity than by the manometric procedure.

Catechol-*O*-methyltransferase can be assayed by measuring normetanephrine or metanephrine formation as described by Axelrod & Tomchick (56). These methylated derivatives are determined by their native fluorescence after solvent extraction from the tissues. Axelrod & Weissbach (23) have described an enzyme (hydroxyindole-*O*-methyltransferase), present in pineal glands, which *O*-methylates a variety of 5-hydroxyindole compounds. N-Acetylserotonin, the most active substrate, is converted to the hormone melatonin, the lightening agent discovered by Lerner (21). The enzyme reaction can be studied by measuring melatonin formation. After extraction of the incubation mixture with chloroform, the organic phase is washed with water to remove substrate, and the chloroform is evaporated to dryness under a stream of hot air. The residue is dissolved in 3 N HCl, and assayed in the spectrophotofluorometer.

Gas Chromatography

Chromatographic procedures have been widely applied in pharmacological research to separate organic compounds. Paper and column chromatographic methods are commonly used for separating drugs and their metabolites, biogenic amines, natural and synthetic steroids, and many other compounds of interest to the pharmacologist (57). However, since these methods are relatively tedious, their efficiency and usefulness to the investigator are reduced. Since chromatography may be considered essentially as the distribution of a solute between two immiscible solvent phases, one stationary and the other mobile, it is not surprising that a chromatographic method has been developed in recent years in which the mobile phase is a vapor and the stationary phase is a nonvolatile liquid dispersed on the surface of an inert solid carrier.

The theory of gas-liquid chromatography (GLC) will not be discussed in this review. Many fine discussions of theories and techniques are available (58 to 62). Very briefly, the apparatus consists of a long length of small diameter tubing packed with an inert carrier such as an infusorial earth (Celite). The surface of the carrier is coated with a thin film of a nonvolatile liquid which serves as the stationary phase. The movable phase consists of a gas stream, usually nitrogen, helium, or argon, which flows through the column at a predetermined rate. The column and the gas stream flowing through it are maintained at some constant temperature which may be varied from room temperature to several hundred degrees. At the outlet end of the gas stream, a detecting device senses and records the minute quantities of compounds present in the effluent gas stream.

Experimentally, a small sample (usually a few microliters) of the un-

known mixture is dissolved in a suitable organic solvent and added to the gas stream through a sample port. As the components of the unknown mixture are volatilized they are swept through the column and distributed between the gas stream and the nonvolatile liquid phase. By varying parameters such as stationary phase, column length, working temperature, and rate of gas flow, combinations can be obtained which will effect a separation of the components of a given mixture. As each substance reaches the detecting device, a signal is produced, which can be recorded, giving the location of the substance in the effluent gas. In most instances, not only qualitative, but also relatively quantitative information can be obtained from the results, especially if a recording device is used in conjunction with the detector. By comparing an unknown mixture with the results obtained for standard compounds under the same conditions, the components of the mixture may be identified. If an integral detecting device is used, the areas under the individual peaks represent the relative quantities of each compound present in the mixture.

Applications of GLC.—Perhaps the first use of GLC was that described by James & Martin in 1952 (63). They separated the straight chain aliphatic acids containing from 1 to 12 carbon atoms, using nitrogen as a carrier gas with a stationary phase of a silicone oil containing stearic acid distributed on a diatomaceous earth carrier. Since that time, many papers have been published on the applications of GLC. However, until very recently, its use was largely restricted to petroleum chemistry and related fields where separation of hydrocarbon compounds was desired.

The first applications of GLC of interest to the pharmacologist were in the field of fatty acid analysis. Procedures were developed for the separation of long chain aliphatic acids present in animal tissues as free fatty acids and glycerol esters (64, 65). In general, the acids are extracted from tissues, esterified to their methyl esters, and the esters separated by a GLC procedure. These techniques have been used to study the effect of drugs on lipid mobilization and transport (64, 66, 67).

Steroids.—In any study of endocrine function or cholesterol metabolism, one of the primary difficulties has been the separation and identification of the steroidal compounds involved. The usual paper chromatographic techniques are often laborious and require long development times under special conditions. By using techniques of GLC, a number of steroids are made readily separable. A general discussion of the separation of steroids by GLC techniques has been made by Vanden Heuvel *et al.* (68). These techniques have been successfully applied to the separation of sex hormones and bile acids (69), adrenal cortical hormones (70), miscellaneous steroids (71 to 73) and the D vitamins (74). More recently, new silicone polymers (75) and polyester liquid phases (76) have been used, which greatly facilitate the separation of various steroidal compounds. For example, the 17-ketosteroids usually excreted in human urine have been separated and quantitatively

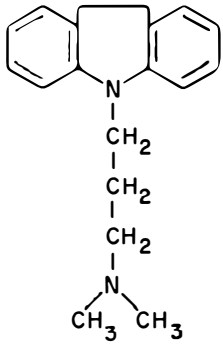
determined by a GLC procedure after hydrolysis of urine and extraction of the steroids with an organic solvent (77). A GLC method has also been described for the determination of cholesterol and squalene (78). This method may be applicable to the quantitative determination of these compounds in blood.

Amino acids and derivatives.—In 1958, Zlatkis & Oro (79) reported the use of GLC techniques to separate a number of amino acids. The amino acids were treated with ninhydrin in a cold alcoholic solution. When this solution was applied to a GLC column at high temperatures, the amino acids were converted on the column to volatile aldehydes which could be separated by the apparatus. This technique has been used successfully to separate mixtures of alanine, α-amino-n-butyric acid, valine, norvaline, leucine, isoleucine, and norleucine (80).

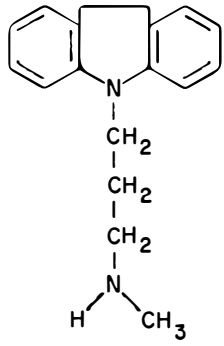
Another technique for GLC separation of amino acids has been reported by Youngs (81). Amino acids from protein hydrolysates are treated with acetic anhydride in n-butanol, and the resulting N-acetylamino acid n-butyl esters are separated by GLC. Quantitative determinations of glycine, alanine, valine, leucine, isoleucine, and proline are possible. Recently Johnson *et al.* (82) have reported the separation of 35 amino acids by a GLC procedure after conversion of the acids to the N-acetylamino acid m-anyl esters. Preparation of the derivatives and chromatographic separation takes about three hours and quantitites of amino acid of the order of 10^{-10} mole can be detected.

The relationship of amino acids as precursors of biogenic amines and of urinary aromatic acids as metabolites of these biologically important compounds has been investigated with GLC techniques. Pisano *et al.* (83) are able to separate microgram quantities of amines such as amphetamine, ephedrine, norephedrine, serotonin, histamine, tryptamine, metanephrine, and normetanephrine by direct GLC. Sweeley & Williams (84) used GLC to separate and quantitatively estimate aromatic acids such as p-hydroxyphenylacetic, homovanillic, hippuric, 3-methoxy-4-hydroxymandelic, indoleacetic, and 5-hydroxyindoleacetic acid from human urine after conversion of the acids to their methyl esters.

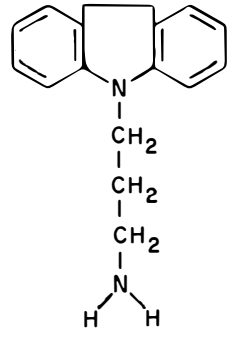
Drugs and miscellaneous compounds.—The techniques of GLC have been used to determine ethanol in blood (85), the nonvolatile acids in cigarette smoke (86) and various chlorinated and organophosphorous insecticides (87, 88). A particularly interesting application of GLC techniques to drug metabolism can be seen in the recent studies of imipramine by Gillette and coworkers (89). This drug undergoes stepwise demethylation *in vivo*, first to a highly active metabolite (desmethylimipramine) and then to an inactive primary amine. These biotransformations affect only one small part of the chemical structure, and paper chromatography was not suitable for separation. However, the tertiary, secondary, and primary amines were readily isolated by solvent extraction and easily separated by GLC.



IMIPRAMINE



DESMETHYL-IMIPRAMINE



DESDIMETHYL-IMIPRAMINE

New Developments in Radioisotope Techniques

In the past five years, two major advances in radioisotope technology have opened new vistas to the investigator in pharmacology. These two developments, tritium labeling by exchange with gaseous H_3 (the Wilzbach technique) and the internal liquid scintillation counting technique, are mutually dependent and each of them will be treated individually.

Tritium gas exposure labeling.—Since the first reported use of this technique (90), many papers have been published on the preparation of tritium labeled compounds for tracer studies. In general, the procedure involves the exposure for several days of an unlabeled compound to curie amounts of tritium gas. Although the procedure has been used to label a wide variety of compounds, its efficiency varies considerably. For example, the fraction of tritium incorporated per day has been reported to range from 10^{-6} to 10^{-2} (91). In an attempt to improve the efficiency of the labeling, acceleration procedures, such as exposing the reaction vessel to ultraviolet light (92) or to x-radiation (93), have been used. Passage of an electric discharge through the reaction mixture has also been examined as a possible accelerating force (94 to 96).

A problem which occurs in tritium gas exposure labeling is that of product purification. In any application of this technique, a large number of labeled by-products are formed. These by-products have high specific activities and often differ only slightly in chemical structure from the parent compound. Indeed they are often the products of racemization, or isomerization reactions. Thus the isolation of a radiochemically pure product may be difficult.

The uses of H^3-labeled compounds in research problems of interest to pharmacologists have been many and varied. Several studies on the disposition and fate of H^3-catechol amines have been reported. For example, Axelrod

and co-workers have studied the physiological disposition of tritium-labeled epinephrine, and metanephrine (97) and tritium-labeled norepinephrine (98) have been examined, using doses in the physiological range. Dengler *et al.* (99) have examined the uptake of catechol amines by various tissues using tritium-labeled compounds of high specific activity. The advantage of tritium-labeled compounds for these studies lies in the high specific activities available, which permit the administration of quantities small enough not to upset the normal body levels.

The applications of H^3-labeled compounds in the field of steroids have been more numerous. For example, the hydrocortisone derivative triamcinalone (9α-fluoro-11β,16α,17α,21-tetrahydroxy-1,4-pregnadiene-3,20-dione) has been successfully labeled by gaseous exchange (100), and its distribution and excretion have been studied (101). Similarly, corticosterone-1,2-H_3 is also available and has been used in determining the rate of metabolism of this steroid (102). Once again the high specific activity (670 mC/mM) permits the use of small quantities. For example, 5 μc of activity are contained in only 2.5 μg of steroid.

Many therapeutic agents have been labeled with tritium by the gaseous exposure technique. Meprobamate (2-methyl-2-propyl-1,3-propanediol dicarbamate) (103), digitoxin (104), and insulin (105) have been labeled with H^3 by this technique. One of the most interesting studies is that recently reported by Woods and co-workers (106, 107). Normorphine was labeled with tritium by the Wilzbach exposure technique. After purification, part of the yield was used to study the disposition and excretion of this compound, while another fraction was chemically converted to tritium nuclear-labeled morphine and the disposition of this compound was also investigated.

Liquid scintillation counting.—The technique of counting low energy β-emitters by using internal liquid scintillation counting is a relatively recent one (108 to 113). The β-emitting compound is dissolved or suspended in a medium which has the property of emitting photons of light as the β-particles are absorbed. Sensitive photomultiplier tubes "see" these photons as individual events. The entire photodetection system is usually contained in a deep-freeze to permit operation at temperatures of 0°C to decrease background "noise" in the photomultipliers. The output of the photomultipliers is passed into a counting system which records the number of events.

In general, the labeled compound under investigation is dissolved or suspended in a "cocktail" consisting of a solvent, a primary solute, and a secondary solute. The energy of the β-particles is transferred by the solvent molecules to the primary solute. Photons of energy emitted by the primary solute are usually too far in the ultraviolet region of the spectrum to be detected directly, and so the secondary solute or "wavelength shifter" is added. These compounds have the property of absorbing the short wavelength photons and emitting photons of longer wavelength light, commonly around 3800 A. A typical "cocktail" for C^{14}- or H^3-labeled organic compounds con-

sists of 100 mg of 1,4-bis-2-(5-phenyloxazolyl)-benzene as secondary solute, 4 g of 2,5-diphenyloxazole as primary solute, dissolved in one liter of toluene as solvent. This system counts C^{14} with an efficiency of about 80 per cent and H^3 with an efficiency of about 20 per cent. Many other "cocktail" mixtures have been developing for counting various types of compounds, depending on solubility characteristics.

A difficulty inherent in liquid scintillation counting is the absorption of energy ("quenching") by extraneous compounds present in the scintillation mixture. Quenching effects may be produced by colored compounds (which absorb the light emitted by the phosphor) or by compounds which have an energy of activation lower than the phosphor. The latter category includes many carbonyl compounds, amines, halocarbons, and heterocyclics. Quenching by colored compounds is even more serious and makes it difficult to count samples containing yellow and red materials except at very high dilutions. A technique commonly used to correct for quenching is to recount all samples after the addition of a suitable internal standard ("spike") and then to correct the unknown counts for the efficiency of the standard.

Liquid scintillation counting has a very great advantage in radioisotope studies since it is possible to discriminate between two different nuclides of different β-energies. For example, there is a difference in the average energy of the β-emissions from C^{14} (.155 Mev) and H^3 (.018 Mev). This difference is reflected in the light output of the phosphor and in the signal from the photomultipliers. The discriminator circuits currently used can easily separate the C^{14} and H^3 pulses and the two nuclides can be simultaneously determined in one sample. A similar technique can be used for other nuclides having β-energies of different values.

Applications of liquid scintillation counting techniques.—Since the applications of this technique are far too numerous to review in any detailed manner, we have selected some specific applications which illustrate the wide variety of available "cocktail" mixtures and counting techniques.

Since the scintillation phosphors are nonpolar compounds and must be dissolved in a nonpolar organic solvent (114), highly polar substances such as proteins or amino acids do not dissolve in significant quantities. For C^{14}-labeled compound, a method has been described by Hayes *et al.* (115) in which a suspension of the labeled compound in a nonpolar cocktail is counted directly. However, this method is not suitable for the assay of H^3-labeled compounds.

Passman *et al.* (116) have reported the use of a methanolic solution hydroxide of Hyamine (*p*-diisobutyl cresoxy methoxy ethyl dimethyl benzyl ammonium chloride monohydrate) to absorb $C^{14}O_2$ and permit the measurement as a complex, soluble in toluene-methanol. More recently, Vaughan and co-workers (117) have applied the Hyamine complexing technique to solubilize H^3- and C^{14}-labeled amino acids and proteins, permitting the use of liquid scintillation counting of these compounds.

Steinberg (118) has described several techniques for counting aqueous solutions, using solid scintillators such as anthracene crystals and plastic scintillator filaments. In these systems, the solution containing radioactivity is merely poured into a vial packed with the solid scintillator. Efficiencies were reported for H^3 (0.5 per cent), C^{14} (16 to 20 per cent), Ca^{45} (49 to 93 per cent), P^{32} (56 to 78 per cent), and I^{131} (46 to 58 per cent).

The most recent innovation permitting the successful use of liquid scintillation counting for polar substances is that of Bray (119). He has successfully used a complex cocktail of naphthalene 2,5-diphenyloxanzole and 1,4-bis-2-(5-phenyloxazolyl) in a dioxane-methanol ethylene glycol solvent to count aqueous solutions. As much as 3 ml of aqueous solution will remain in solution with 10 ml of this cocktail at −8°C.

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