APPLICATION OF NEW ANALYTICAL TECHNIQUES TO PHARMACOLOGY

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Increasingly sensitive and systematic probes into the microchemistry at the receptor and cellular level in a physiological system continue to demonstrate the complexity of such an environment. The awareness that minor constituents can play a major role in mediating physiological responses has resulted in even greater demands on analytical methodology. The investigator, realizing that his "model" is no better than his methodology, seeks to isolate or measure selectively the concentration of a specific drug or hormone that is available to target tissue. The central theme of this chapter emphasizes new developments in the purification of biological samples that may contain labile molecules, detection of minor constituents with great specificity, and fixation of tissue to retain or more closely represent in vivo concentrations of certain heat stable drugs and hormones.

Affinity chromatography, based on the biological principle of immunochemistry, can be used as a single-step method of isolating or purifying enzymes and other macromolecules with high retention of biological activity. Applications are described for the isolation of a glucagon receptor as well as purification of acetylcholinesterase, tetrahydrofolate dehydrogenase, and various cell types. This technique also offers the possibility of selectively isolating trace quantities of a drug or specific hormone from biological fluids.

Liquid chromatography holds new promise for the preliminary purification of biological samples prior to quantification by other techniques. Several new developments in high-pressure liquid chromatography permit better interaction between sample and partitioning phases resulting in faster, more efficient separations comparable to those obtained in gas-liquid chromatography. Examples of separating classes of benzodiazepines, coumarins, steroids, and vitamins by high pressure liquid chromatography are described as well as applications of reversed-phase and gel permeation chromatography.

Multiple ion detection (MID) is described as an adjustable selective detector for biologically important molecules that are suitable for vapor phase analysis. The usefulness of this technique is demonstrated with the analysis of a complex biological sample (urinary catecholamine metabolites) that overwhelmed a flame ionization and electron capture detector, but yet was amenable to MID. Another application of MID with stable isotopes is described in the context of prostaglandin analysis. New developments in on-line data acquisition and oscilloscopic display by computer are contrasted with conventional oscillographic recording of MID data.

Finally, an improved method of animal sacrifice is described that arrests enzymatic activity in vivo and thereby provides tissue specimens that contain levels of heat-stable hormones, such as cyclic AMP and acetylcholine, and drugs that closely approximate those in the living animal. Microwave irradiation also facilitates the dissection of intricate organs, such as the brain, for individual analysis of discrete components. Furthermore, sacrifice by microwave irradiation significantly reduces pre-sacrifice stress and circumvents decapitation artifacts. This technique of animal sacrifice is revolutionary in that it may eliminate or at least reduce many of the nonspecific hormonal variations that sometimes mask subtle drug-induced responses.

AFFINITY CHROMATOGRAPHY

Enzymes and other biological macromolecules can be isolated and purified by affinity chromatography through a process governed by their biological interactions rather than by slight differences in physical partitioning between two phases as in conventional chromatography. Physiological specificity is achieved in nature through localized molecular arrangements, which have been termed "active sites" in enzymes and various tissues. In affinity chromatography (1–5) this type of natural selectivity is used advantageously by chemically attaching to an insoluble polymer or gel a given compound that will bind specifically and reversibly the substance of interest. Usually an inhibitor of an enzyme or a ligand for a macromolecule is covalently bound to an otherwise inert polymer or gel, which then serves as a "chromatographic" column packing. When a mixture is passed through the column, the specific enzyme or macromolecule is isolated or retained, since other enzymes have no appreciable affinity for the inhibitor, which is bound to the insoluble polymer or column material. A high degree of purification is thereby achieved in a single operation. Typically, more than half the enzyme applied to the column is recovered and 60–90% of the biological specific activity is also retained (6–9).

The greatest difficulty in using affinity chromatography for purification of a given enzyme or macromolecule may be in coupling a suitable inhibitor or ligand to an inert polymer without altering the chemical nature of the potential binding site. The solid matrix (3, 10) used in many recent applications is Sepharose (Pharmacia), a "beaded" form of agarose, a cross-linked dextran characterized by its highly porous structure. Sepharose has minimal interaction with proteins and forms a loose, porous network that facilitates passage of macromolecules and retains favorable flow properties normally associated with column chromatography. Most often the Sepharose is activated (4, 11) by treatment with cyanogen bromide, which produces a derivative that can be coupled to unprotonated amino groups on the inhibitory analog. Attachment of inhibitors and ligands to agarose and polyacylamide beads through other types of bonds (e.g., carboxyl, phenolic, or imidazole groups) has also been described (3–5). Porous glass beads (12) have been used successfully to immobilize chymotrypsin and β-galactosidase. The glass beads were treated with a 2% solution of 3-aminopropyltriethoxysilane in acetone to produce aminoalkylsilane glass. Gluteraldehyde was then used to

link chymotrypsin, β-galactosidase or peptidyl-L-amino-acid hydrolase (an inhibitor of carboxypeptidase A) to porous glass (12). Enzymes that are covalently attached to such inorganic supports have greater temperature and operational stability than those coupled to organic matrices (13).

In the preparation of any insoluble matrix for affinity chromatography, it is important that the inhibitor or ligand groups be sufficiently distant from the primary structure of the polymer matrix to minimize steric interference with the reversible binding processes. Relatively short hydrocarbon "extensions" (3, 14, 15) can be interposed between the ligand and agarose backbone to minimize steric interference.

Once an appropriately substituted matrix or gel has been prepared, mild conditions must be sought to elute the bound enzyme or macromolecule without denaturing it. This is usually achieved by altering the electrolyte concentration or pH, or by addition of some free, competitive substrate or inhibitor. The latter technique is generally preferable. For example, in the purification of acetylcholinesterase (15), when the enzyme is eluted by increasing ionic strength, it is contaminated with other proteins; however, elution of the enzyme by the addition of decamethonium produces a pure fraction of acetylcholinesterase. In general, after complete elution, the column may be reused.

Glucagon receptor interaction in liver cell membranes has been studied with an insoluble glucagon-agarose derivative (14). This conjugate should be useful in investigations of the functional and structural relationships between glucagon receptors and adenyl cyclase activity in liver membranes. Since the glucagon-agarose derivatives strongly interact with adenyl cyclase receptors in the liver cell membrane (14), affinity chromatography using this phase should be useful in the isolation of membrane receptor structures for glucagon. Insulin receptor interactions in isolated adipose tissue cells (16) have been investigated with an insulin-agarose derivative. These studies were possible because insulin covalently bonded to agarose beads retains its ability to modify several metabolic functions of adipose tissue (16). Purification of acetylcholinesterase (15), thrombin (7), and tetrahydrofolate dehydrogenase (8, 9) by several hundred-fold should facilitate the elucidation of the active sites on these enzymes, which will have important implications in pharmacology. Similarly, purification or isolation of tryptophan hydroxylase by efficient and gentle methods employed in affinity chromatography would surely advance efforts to study the regulation of serotonin synthesis.

Affinity chromatography is well suited for research involving the isolation of receptors for various drugs and hormones. If the drug, hormone, or appropriate molecular analog can be bound covalently under suitable conditions to an inert, solid matrix, the resulting insoluble material may be used as a column packing to isolate receptor-macromolecules for further study. This approach was taken in the isolation of thyroxin-binding globulin (TBG) from human serum using Sepharose to which l-thyroxine had been covalently bound (17). Previous methods for the purification of this hormone-transport protein involved laborious procedures with rather poor yields. The use of affinity chromatography in this case provided

a simple two-step method for obtaining TBG directly from human serum in 18–37% yield (17).

An analogous approach to isolating estradiol binding proteins from human serum involved first the preparation of an estradiol-Sepharose derivative (3). In this case it was possible to elute the strongly adsorbed serum estradiol-binding protein by cleaving the estradiol-Sepharose bond with mild base or with neutral hydroxylamine (1*N*), thus avoiding the use of protein denaturants.

Various cell types are often distinguished by the presence of specific receptors in the plasma membranes. Similarly, appropriate proteins covalently bound to agarose under well-defined conditions can be used to isolate specific cell types from biological samples (5, 18). Cuatrecasas (19) has used an insulin-agarose matrix to isolate fat cells and an aglutinin-agarose conjugate to scavenge erythrocytes.

On the other hand, for analytical purposes, an immobilized enzyme or ligand might be conditioned so that it would bind, but not metabolize, a specific drug or hormone and thereby isolate or "extract" it from a sample of biological fluid. In principle, large quantities of biological fluid could percolate through a column containing the enzyme-matrix conjugate and only the specific drug or hormone would be retained because of its high affinity for the immobilized enzyme. Affinity chromatography employing an immobilized macromolecule would be useful in the analytical pharmacology laboratory where trace amounts of drug or hormone had to be isolated from large quantities of biological fluid. Methodology in prostaglandin research would be greatly improved by the development of a means of isolating trace quantities of prostaglandins (even as a group) from biological fluids or tissue homogenates.

Many previous attempts at receptor isolation have failed, principally because the method employed usually denatured the receptor. Since the technique of affinity chromatography is based on very specific, yet gentle and reversible binding that mimics natural, immunological interaction, the biological activity of many sensitive enzymes is maintained throughout the isolation and purification process. Many of these isolated enzymes, in turn, provide the possibility of being immobilized by attachment to an inert matrix, which may be used to isolate specific hormones or drugs from biological samples. Affinity chromatography should have a great impact on research in enzyme mechanisms and on the development of improved analytical methodology in this decade.

Liquid Chromatography

Many complex biological samples in analytical pharmacology are amenable to liquid chromatography. Several variations of this oldest established method of chromatography are available, and this section describes applications of conventional, high pressure, reversed-phase, and gel permeation liquid chromatography. The potential of these techniques (20) in analytical work has increased greatly in recent years both for complete separation and quantification of some drugs and hormones and as a means of "coarse" or preliminary purification of biological samples that are too complex for complete analysis by other tech-

niques. Furthermore, some drugs and hormones cannot be suitably derivatized or are too labile for analysis by gas-liquid chromatography, yet are suitable, underivatized, for analysis by some form of liquid chromatography.

New developments in instrumentation and in chromatographic matrix and phase design have made high-speed, high-resolution, high-capacity liquid chromatography a reality. Perhaps the most significant advances have been made in the manufacture or preparation of supports for high speed liquid chromatography as reviewed recently by Kirkland (21). These supports have "controlled surface porosity," which provides a relatively large surface area with a solid, impervious core. Since there are no deep crevices or channels in these beads, a thin film of sorbent can be uniformly dispersed over the bead surface, resulting in rapid mass transfer and partitioning of the sample under analysis.

Equally important is the development of "permanent" (22) stationary phases prepared by chemically bonding organic polymers to the controlled surface-porosity support. These new column packings have nonextractable, thermally and hydrolytically stable, organic coatings. Utilization of these bonded-phase supports in liquid chromatography eliminates the necessity for pre-equilibration of the carrier with the stationary phase; also the sample is not contaminated with the stationary phase. These packings with chemically-bound stationary phases are ideal for gradient elution and flow-programmed liquid chromatographic separations. Good column efficiency is maintained at higher flow rates because of the more homogeneous distribution of the chemically-bound organic stationary phase (23). In many cases, use of the controlled surface porosity bead packings permits the preparation of liquid chromatographic columns (20), which are equivalent in performance to packed gas chromatographic columns.

Analytical liquid-chromatography can be accomplished on columns having an internal diameter of 2 mm, which can accomodate samples ranging from 10–100 micrograms. Larger samples (up to 5 mg) require preparative columns having an internal diameter of 8 mm to achieve comparable resolution of the constituents. Column efficiency can be improved by the use of long, narrow bore columns, but these have a very large pressure drop. However, several commercial instruments are available that can force a variety of eluates through liquid chromatographic columns at pressures up to 6000 pounds per square inch.

The main problem in instrumentation for high pressure (thus high speed) liquid chromatography is a sensitive detection system (24). The ultraviolet detector is sensitive to about 10^{-7} gm/ml for those compounds that contain or can be derivatized with a suitable chromophore. The refractometer detector is a more general and rugged detector, but cannot be used with gradient elution, is temperature sensitive, and can detect only about 10^{-5} gm/ml. Several other, more sensitive detectors (24) are available, but in some cases they are prohibitively expensive.

There are increasing reports of the use of high pressure liquid chromatography in drug analysis such as in the separation of benzodiazepines (25), for example. This analytical technique has also been used in studies involving the urinary

excretion of the diuretic metolazone (26); the residue of chloroform extracts of urine samples were analyzed directly on a 6 ft × 1/8 in Durapak (Carbowax 400/Porasil C) column. Many hormones are also amenable to high pressure liquid chromatography. Examples of all major types of steroids, as well as conjugates such as digoxin, digitoxin, and estradiol glucosiduronic acid (27) have been analyzed by high speed liquid chromatography from modern solid-core supports coated with appropriate stationary phases. In this case, the UV detector of fixed wavelength (254 nm) gave a lower limit of detection of 10^{-6} gm for those steroids with no electronic conjugation (androsterone), 10^{-8} gm for those with a conjugated system (testosterone), and 10^{-9} gm for those in which the carbonyl of the steroid was derivatized with 2,4-dinitrophenylhydrazine, e.g., androsterone-DNPH (27).

The analysis of biological samples for nucleic acid components by high-pressure liquid chromatography is well established (28, 29). This technique has also been used to separate purine and strychnos alkaloids of pharmacological interest (30). The analysis of analgesics by high speed liquid chromatography (31) obviates many procedural problems involving extractions of tablets with various solvents followed by "batch" determinations by ultraviolet or refractive index measurements. Application of high speed liquid chromatography to the analysis of antibiotics has had only limited success although the separation of Rifampin derivatives on a polyamide column eluted with hexane : ethanol (3 : 1) has been reported (32). Use of an ion exchange system is more universally applicable, however; for example, several of the carbohydrate antibiotics (Kanamycin, Gentamycin, and Neomycin) can be chromatographed at pH 9.2 with a phosphate buffer in the range of 0.006 ***m*** to 0.012 ***m*** (32).

Vitamin analysis is now amenable to liquid chromatography (33). Water-soluble vitamins are best chromatographed by ion exchange chromatography while fat-soluble vitamins are best separated by the reversed-phase technique employing a polar water-alcohol solvent over a nonpolar stationary phase. Separations of several members of the vitamin A, B, C, and K families have been reported (33). In the analysis of tablets or capsules for multivitamins, difficulties with fat-soluble vitamins are minimized by extraction with dimethyl sulfoxide and then hexane (33). The water-soluble vitamins are removed by simple water extraction and injected into the liquid chromatograph without further preparation.

In reversed-phase partition chromatography, the stationary phase is non-polar and the mobile phase is polar. This type of chromatography is especially useful in the analysis or purification of compounds that are lipid (or hydrocarbon) soluble; furthermore, this technique can be applied to any sample that is sparingly soluble in water. One reversed-phase method for steroids (34) uses a water-insoluble amine stationary phase and a water/methanol mobile phase. Another example of reversed-phase high-pressure chromatographic separation of some coumarin derivatives (31) is shown in Figure 1.

With attention to proper techniques (35) of operation the high performance and stability of an analytical liquid chromatographic system can be maintained

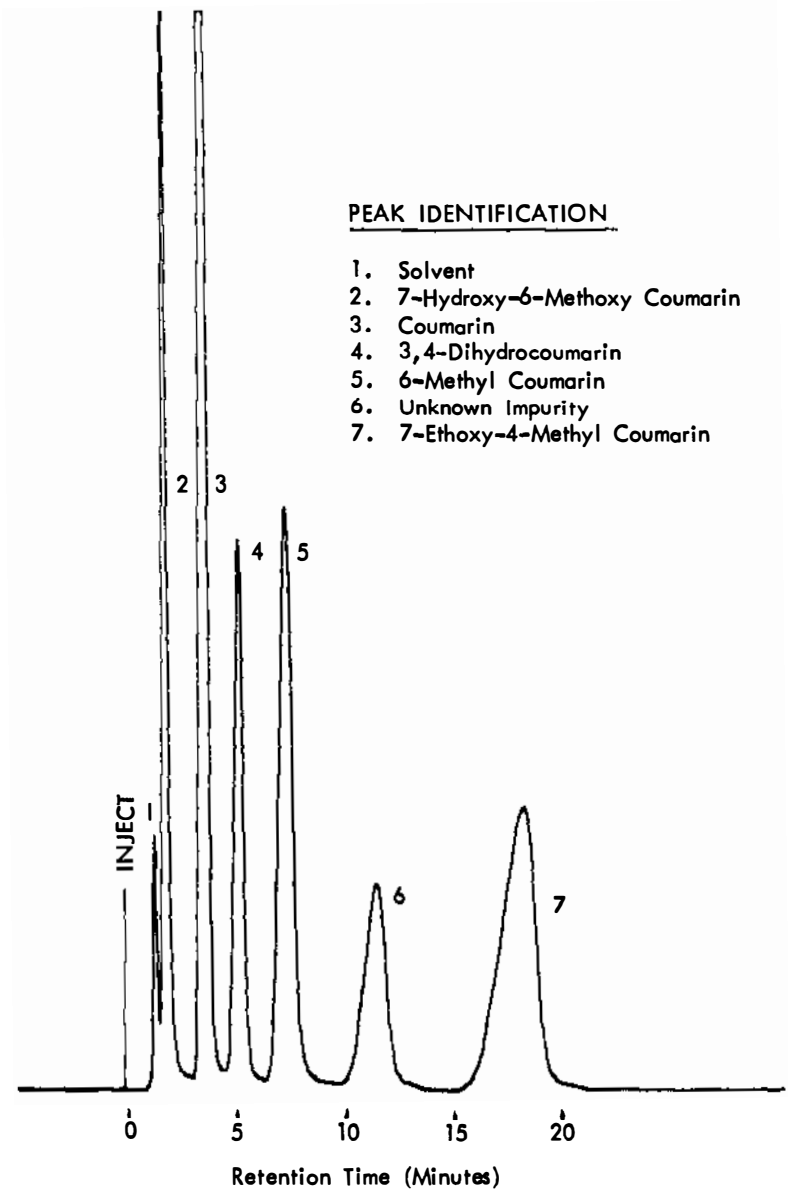

FIG. 1. Separation of coumarin derivatives by high pressure liquid chromatography: 1 m × 2.1 mm 1% cyanoethyl silicone on Zipax® eluted with H_2O at 1200 psig (ref. 31). Reprinted by kind permission of Dr. J. A. Schmit and of John Wiley & Sons, Inc.

for several months. In some cases, final quantification may be more appropriate by a more sensitive technique, but rapid preliminary purification of a biological sample by an efficient, preparative high-pressure liquid chromatograph should find useful application in many areas of pharmacology.

Gel permeation chromatography (36) is a liquid-solid column chromatographic method of separating compounds according to differences in molecular size. As the sample percolates through the column the porous, uncharged, spongy gel molecules too large to diffuse into the porous matrix pass through the column bed very rapidly through the space between gel particles, and thus are quickly eluted. Molecules small enough to penetrate the matrix structure of the gel are delayed or "retained" due to their longer migration path through the interior channels within the gel particles. The smaller molecules are thus eluted in order of decreasing molecular size.

Gel filtration (or permeation) methods have been devised to accomodate organic (36) and aqueous (37) solvents. The technique is a simple and rapid means of preliminary purification for biological samples in which it is desirable to separate molecules under molecular weight 1000 from such macromoles as enzymes and other proteins. The technique is limited to those samples and eluents having low viscosity. Aromatic substances are often sorbed strongly, which results in poor recoveries of this class of compounds.

Multiple Ion Detection in Vapor Phase Analysis

Rarely does an analytical technique share both high sensitivity and "selectable" specificity. Multiple ion detection (MID) with a combined gas chromatograph-mass spectrometer (GC-MS) utilizes the mass spectrometer as a detector to monitor specific ions that characterize the substance of interest as it emerges from the gas chromatograph. The details of GC-MS are described by Jenden in another chapter (38) of this book. Other reviews of GC-MS with emphasis on the fundamentals of carrier gas separators (39) and instrumentation (40) have appeared recently. Innovations in data acquisition for MID with qualitative and quantitative applications are described here.

In MID the MS is operated to monitor only a few selected ions, which are representative of the compound of interest (41). Since the mass spectrum of a given compound is characteristic, though not totally unique, a high degree of specificity is obtained with the mass spectrometer serving as a detector for a gas chromatograph.

MID is a useful qualitative technique in analytical pharmacology. For example, in the development of methodology for normetanephrine (NMN) as the pentafluoroproprionyl (PFP) derivative by GLC with electron capture detection (ECD), injections of a biological extract (unpublished, this laboratory) into the GLC often overwhelmed the ECD with contaminants. At that stage, only by MID was it possible to ascertain whether NMN-PFP was, in fact, present in the treated, biological extract. The mass spectrum of NMN-PFP (42) indicates two prominent ions at m/e 458 and 445 in the ratio of 2 : 1. The GC-MS was adjusted to monitor m/e 445 and 458. The complexity of this particular biological extract is represented

in Figure 2, which shows the parallel recordings of the total ion current (TIC) and the MID unit during the gas chromatographic analysis. The upper panel of Figure 2 is the TIC trace, which simulates a flame ionization detector on a GLC; that is, the TIC responds to all the material emerging from the GLC column. The lower panel of Figure 2 is a photograph of the oscillographic recording of MID, which responds only to those compounds that can produce an ion of m/e 445 and/or 458. The time scale of the upper and lower panels is identical. Just prior to the analysis represented in Figure 2, a pure standard of NMN-PFP was injected into the GC-MS to establish the retention time as 3.7 min under the existing GLC conditions. As can be seen in the upper panel of Figure 2, there is no discernible peak at the appropriate retention time on the recording of the TIC because this transducer is overwhelmed by the multitude of other substances that emerge from GLC with NMN-PFP. In the bottom panel of Figure 2, it can be seen that several substances have emerged from the column that can produce an ion of m/e 458 with a trace of m/e 445, but only at 3.7 min do both ion current profiles rise and fall in the ratio of 2 : 1, which confirms the presence of NMN-PFP in the complex biological extract. It can be seen that the vast majority of the "contaminants" in the urine extract cannot produce the ions of 445 and 458 and thus do not overwhelm the MID unit. The aliquot of extract injected in this case contained approximately 5 ngm NMN-PFP, which is at least 1000 times more than can readily be detected by MID (unpublished results in this laboratory), but a large sample had to be used in this case to produce a demonstrable profile on the relatively insensitive TIC recorder.

The feasibility of quantitative MID has been demonstrated with prostaglandin standards (43, 44). Quantitative analysis of drugs and hormones using stable-isotope-labeled internal standards rather than radioactive analogs should have important applications in clinical pharmacology (45). This technique has been used to measure nortriptyline (46, 47), bile acids e.g., chenodeoxycholic acid (48), and 5-hydroxyindole-3-acetic acid (49). Picomole levels of norepinephrine and dopamine have been measured (50) by a similar method using α-methyl derivatives of these amines rather than deuterium-labeled analogs as internal standards.

Interpretation of MID results, in either qualitative or quantitative analysis of complex biological samples for traces of the compound of interest, is often ambiguous when conventional oscillographic recording is used. It is sometimes difficult to distinguish two or three ion profiles on an oscillographic MID data record. This ambiguity arises because the recording medium, namely a light beam, cannot be conveniently interrupted as the accelerating voltage is switched between preset values. The resulting "streaking" effect makes it difficult (although it is possible, in principle) to identify the various ion profiles, especially when subnanogram quantities of material are being detected. The profiles are defined by the endpoints of the transient sweeps on the oscillographic record as seen, for example, in the lower panel of Figure 2 and the upper left panel of Figure 3.

On-line acquisition, reduction, and display of MID data by a small laboratory computer (PDP-12) greatly facilitate interpretation, unequivocal identification

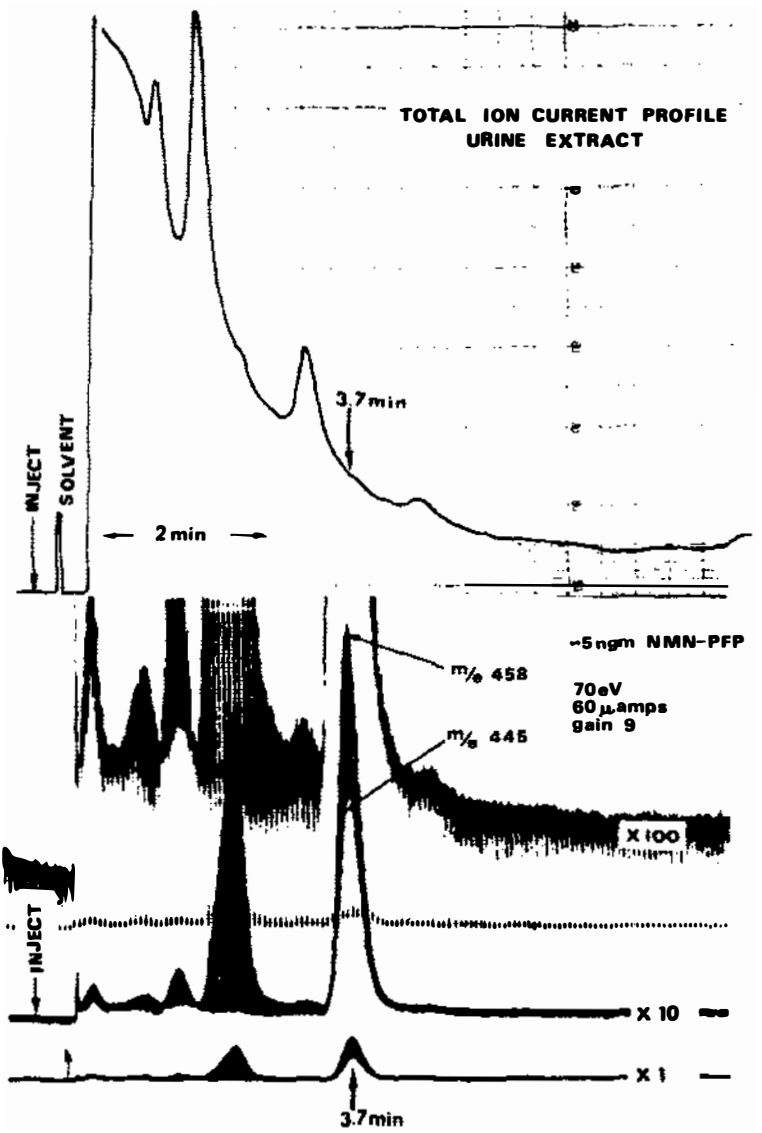

FIG. 2. Comparison of 2 types of GC–MS output from nonspecific totalion current detector (upper panel) and multiple ion detector (lower panel) recorded in parallel during analysis of biological extract for normetanephrine as described in text.

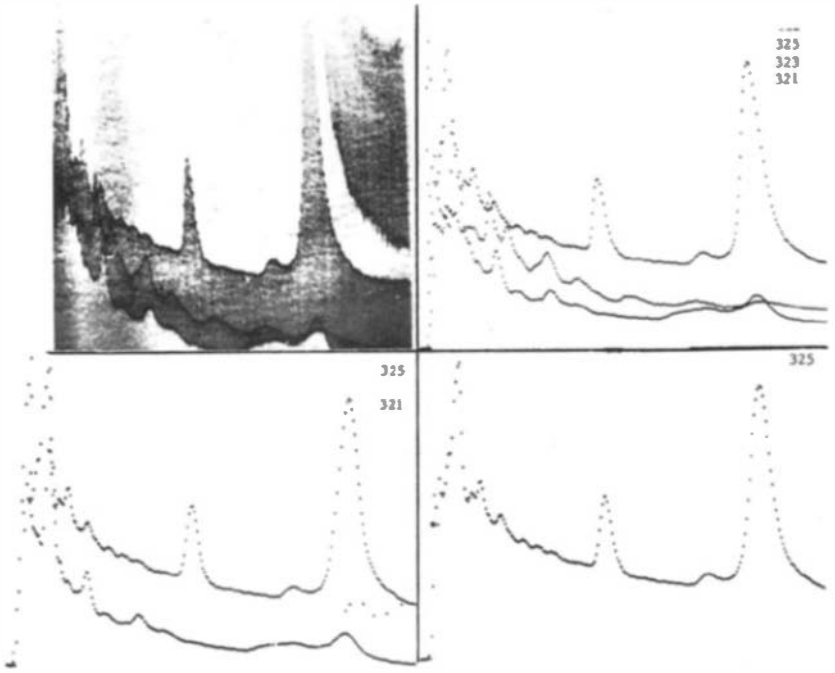

FIG. 3. Comparison of conventional oscillographic recording (upper left panel) and oscilloscopic display by computer of same MID data; sequence described in text.

of various ion current profiles, and calculation of peak areas (51). A comparison of oscillographically recorded and computer-acquired MID data from the same sample is made in Figure 3. These data result from the co-injection of 900 picograms of prostaglandin B_2, methyl ester, trimethylsilylether (PGB_2-ME-TMS) and 9 nanograms of deuterium-labeled d4-PGB_2-ME-TMS in the presence of 1/10 the extract from 5 ml human plasma; these two derivatives are characterized by ions of m/e 321 and 325, respectively. The ion of m/e 323 was also monitored as an index of PGB_1-ME-TMS. The upper left panel of Figure 3 is a photograph of the conventional oscillographic record in which three different profiles should be discernible. The upper right panel of Figure 3 is a photograph of the oscilloscopic display of the three ion current profiles acquired in parallel from the same sample by the computer. The question remains, however, "which profile is which?" The investigator identifies the various profiles by manipulating switches on the computer console that control the display of each profile and its corresponding m/e indicator. In the sequence going to the lower left in Figure 3, then to lower right, the operator has stripped away the display of the m/e 323 profile and finally that of m/e 321.

Retention times of various peaks may be established by displaying a time

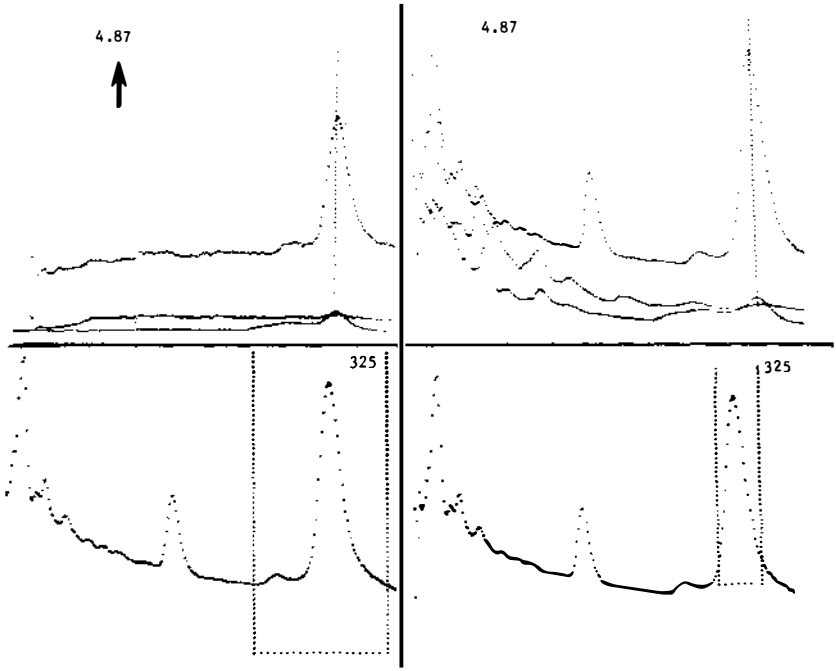

FIG. 4. Photographs of computer-oscilloscope illustrating MID data handling with the retention time-cursor (upper panels) and the peak area calculation procedure (lower panels) for a prostaglandin sample.

cursor on the screen and positioning it at the center of the peak of interest. The retention time to the nearest 1/100th minute can be read from the oscilloscope. In the photograph of the computer-oscilloscopic screen at the upper left of Figure 4, the operator has centered the time cursor on peaks resulting from 0.9 ngm PGB_2-ME-TMS (m/e 321) co-injected with 9.0 ngm d-4-PGB_2-ME-TMS (m/e 325) to establish a retention time of 4.87 minutes (see arrow). The upper right photograph in Figure 4 shows that the same standards co-injected with 1/10 aliquot of the extract from 5 ml human plasma give the same retention time on 2m × 2.5 mm, 1.5% OV-17 on chromosorb-G-HP at 265°C.

Calculation of peaks areas is also facilitated by the computer. In this software routine, a set of three adjustable cursors, which resemble three sides of a rectangle, are displayed on the oscilloscope along with a selected ion current profile as indicated in the lower left panel of Figure 4. The operator adjusts the position of each cursor to the baseline, leading, and trailing edges of the peak, respectively, (lower right of Figure 4) prior to depressing the "C" key on a teletype, which prints the value of the peak area within the designated limits. Small peaks may be magnified on the computer screen to facilitate judgment on positioning the area-limiting cursors. Data acquisition and reduction as described here ensure

accurate identification of ion profiles and eliminate many sources of human fatigue error in area calculation, yet provide for human intervention and judgment.

In Situ Enzyme Inactivation by Microwave Irradiation

Estimation of in vivo levels of hormone and enzyme activity by measurement of these substances in tissue from sacrificed animals is subject to many assumptions and variables. The very process of killing the animal can alter hormone levels (52) and, in some cases, response to the nonspecific effects of stress and sacrifice could override that under experimental investigation. Many substances undergo rapid post-mortem changes such as glucose and lactic acid (53) and brain glycogen (54). Furthermore, the method of sacrifice can affect certain hormones; for example, cyclic AMP rises rapidly following decapitation (55). In most cases, these post-mortem alterations in hormone levels are due to sustained activity of synthesizing or degrading enzymes.

Enzymatic activity can be rapidly arrested in situ by exposure to microwave irradiation (56), which most likely results in heat denaturation of proteins. The principal difference between microwave irradiation and conventional heating is that microwaves penetrate deeply into tissue, thereby uniformly and rapidly heating an entire organ (57). The experimental details of animal sacrifice by microwave irradiation have been thoroughly investigated and reported in cases where the heat-stable hormone of interest was cyclic AMP (55, 58, 59) and acetylcholine (56, 60). Microwave irradiation from a commercially available unit completely inactivated adenyl cyclase, phosphodiesterase, and cholinesterase in rat brain after 15–20 sec exposure (55, 60) as indicated in Figure 5. The levels of c-AMP reported in such studies (55, 58, 59) are thought to be a more realistic estimate of in vivo levels than those results reported from experiments in which the animals were decapitated, and comparable to those obtained after "quick" freezing, a process that requires up to 80 seconds (61) to cool deep portions of the brain. ACh levels following microwave irradiation are generally higher than those found following "quick" freezing or other inactivation techniques (see comprehensive table in Ref. 60). The higher levels of ACh reported in these studies are probably more representative of in vivo levels because microwave irradiation arrests the ChE activity that would otherwise be available for post-mortem catabolism of ACh (60).

While rapid freezing (61) methods do reduce post-mortem enzymatic activity, the process is not irreversible, and furthermore, dissection of complex organs such as the brain, is not feasible (55, 60). On the other hand, since enzyme inactivation by microwave irradiation is irreversible, adequate time can be taken for careful dissection of an organ. It is noteworthy that dissection of rat brain is actually facilitated by irradiation as the then firm tissue readily separates along discrete anatomical boundaries (55, 60).

Pre-sacrifice stress is also reduced with the microwave irradiation technique (55, 60). There is no odor of blood from previously sacrificed animals and the odor from previously irradiated rats is not aversive to other rats since they readily

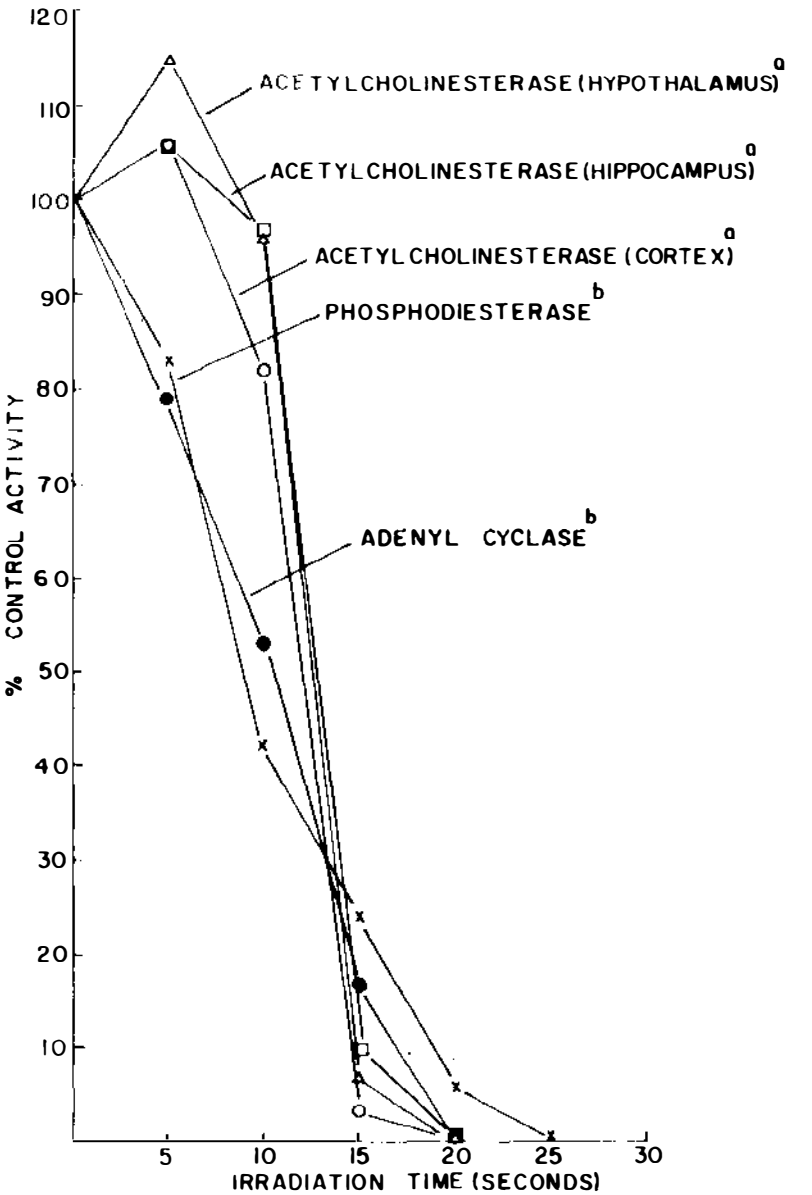

FIG. 5. Plot of residual biological activity after indicated periods of whole-rat exposure to microwave irradiation (1250 watts) for various enzymes. (a, ref. 60; b, ref. 58)

enter the oven chamber immediately after an irradiation (60). Furthermore, the microwave technique requires no close restraint of the animals.

Discrete regions of the brain can be analyzed for c-AMP (58) and ACh (60) following microwave irradiation. Most freezing techniques do not permit accurate dissection of the brain into component parts, a problem that results in "whole brain" studies. Drug-induced alterations of hormone levels in small brain regions could easily be masked or "diluted" by larger amounts of non-responding tissue when the entire brain must be analyzed as a single specimen. A study of tissue fixation by microwave irradiation showed that c-AMP is differentially distributed in discrete regions of the brain (55); the concentration of c-AMP was highest in the cerebellum and brain stem and lowest in the hippocampus and cortex. Thus, for well defined pharmacological studies on the brain it is important to separate accurately the various components of the brain for individual analysis after rapid enzyme inactivation; this can be accomplished by microwave irradiation.

Pre-sacrifice stress and post-mortem enzymatic alteration of hormone levels can be significantly reduced by microwave irradiation sacrifice (55, 60). Analysis of the tissue of animals killed by microwaves thus probably more closely represents those that were present in the live animal. Sacrifice by microwave irradiation circumvents decapitation artifacts (58, 60), irreversibly inactivates enzymes, and facilitates dissection of organs such as the brain. These pioneering studies of microwave sacrifice were accomplished with a commercial version of a microwave oven that operated at a frequency of 2450 mc and had a power output of only 1250 watts (55). Such a device can give results that vary with the size of the animal, because the heating time (time for complete enzyme inactivation) varies inversely with the mass of tissue in such a small oven. This instrumental problem can be overcome by positioning the waveguide for maximum energy transmission to the animal head (62). Using this technique with a 1500 watt (DX-206) magnetron, Stavinoha et al. (62) were able to deliver 1100–1200 watts to the animal head and thereby inactivate enzymes in less than 4 seconds, and effect death in less than 1 second in 100–200 gram rats.

In situ inactivation of enzymatic activity by microwave irradiation should be useful in the analysis of many tissues and physiological fluids in which the hormones and drugs of interest are heat stable. Various prostaglandins have been reported to be present in many tissues at relatively high levels. While many, and perhaps all, tissues have a capacity to synthesize prostaglandins (63), the analytical values reported in many cases may be high because of sustained post-mortem synthesis. Tissue fixation by microwave irradiation followed by quantitative measurement of prostaglandins (43, 44, 64) might reveal more realistic tissue levels of these potent local hormones.

Conclusion

Increasing awareness of the importance for detection and quantification of specific hormones or drugs and drug metabolites in pharmacological studies has forged the basis of several improved analytical concepts. The fundamentals

of some of these new concepts involving sample purification, specific detectors and animal sacrifice have been described in this chapter. Many refinements of these new approaches will be obvious. For example, the potential of high-efficiency, preliminary purification of biological samples by high pressure liquid chromatography prior to quantification of the substance of interest by another, more sensitive technique, such as gas-liquid chromatography with electron capture detection, should not be overlooked. Continued development and refinement of analytical techniques of high selectivity and specificity should accurately accelerate our efforts to unlock some of Nature's secrets.

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