oxide reference solution was stable for about 1 week when prepared in distilled water. However, a change in pH or contamination with inorganic halide would induce rapid conversion of the ethylene oxide to ethylene glycol or ethylene halohydrin. The same concern existed for the sample preparations. Therefore, samples were always chromatographed on the same day that they were prepared.

Precision—Reproducibility of the chromatographic response was about 2% in each case, determined by replicate injections of reference solutions. The variation in results for actual samples was about 5%.

Simplicity—The measurement of the levels of residual ethylene oxide, ethylene chlorohydrin, and ethylene glycol present in pharmaceutical preparations is more of a limits test than a precise quantitation for potency determination. Thus, it is desirable to use rapid, simple, sensitive, and fairly accurate methods for these measurements. The procedures as described satisfy these requirements.

The sample preparation was as simple as possible. All three residuals were extracted simultaneously by a single aqueous extraction.

The chromatographic systems were ideal. Two columns in one dual column instrument provided the necessary instrumentation for estimating all three residuals without changing oven temperature.

Chromatographic time was held to a minimum. Since the highest precision was not required, internal standards were not employed which minimized chromatographic time and permitted a single sample preparation to be employed for all three residuals. Water proved to be the ideal solvent. It provided the highest extraction efficiency, yet did not respond in the flame-ionization detector. If an organic solvent had been required, it would probably have eluted after ethylene oxide and time would have been lost waiting for a large solvent peak to clear the column. With water, this delay was eliminated.

These procedures are versatile and applicable to a large number of aqueous suspensions, aqueous solutions, ointments, and water-insoluble bulk drugs.

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Enzymatic Activity of Pig Heart Mitochondrial Malate Dehydrogenase Monomolecular Films by Surface Exchange Technique

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Abstract □ A technique for studying the catalytic activity of enzymes spread as a film at an air–water interface, by exchanging the subphase under the film to remove unspread enzyme molecules, was developed, and its effectiveness was studied using surface-spread mitochondrial malate dehydrogenase. Mitochondrial malate dehydrogenase formed stable films which gave reproducible π–A curves. The enzyme activity was measured by the oxidation rate of reduced nicotinamide adenine dinucleotide (NADH) in the presence of the substrate oxalacetic acid. Oxalacetic acid and NADH were injected into the subphase. The catalytic activity of the enzyme was dependent on the surface pressure of the film. The maximum catalytic activity was observed at a surface pressure of 4.4 dynes/cm. The activity was higher at intermediate surface pressures than at very low or very high surface pressures. A high bulk catalytic activity was observed in the unstable region, *i.e.*, at a high degree of compression, of the film. The catalytic activity of the surface-spread enzyme was only a fraction of an equivalent amount of enzyme in solution.

Keyphrases □ Catalytic activity—enzymes spread as a monomolecular film at an air–water interface, surface exchange technique developed □ Enzymes—mitochondrial malate dehydrogenase, spread as a monomolecular film at an air–water interface, surface exchange technique developed □ Malate dehydrogenase, mitochondrial—spread as a monomolecular film at an air–water interface, surface exchange technique developed □ Films, monomolecular—of enzymes at an air–water interface, surface exchange technique developed □ Surface exchange technique—enzymes spread as a monomolecular film at an air–water interface

Monomolecular films offer a useful model system for studying the behavior of membrane components at an air–water interface (1). The application of monomolecular films to the study of interactions that may occur at the membranes of cells and cell organelles is a flexible and versatile system whereby numerous effects can be observed. The relative simplicity of this system permits analysis and understanding of the effects that often cannot be observed in the extremely complex environment of living systems.

BACKGROUND

Modification of the film can be employed to gain further insight into cellular function. Since membrane-bound enzymes are situated at interfaces, it must be assumed that they are in a partially unfolded state and that the side chains are oriented in relation to the interface. Monolayer studies also afford a method to study the conformational change effects of proteins by monitoring the surface pressure, since the orientation of the side chains of surface-spread protein would be expected to vary with the surface pressure of the film. A dependence of the catalytic activity on surface pressure was reported for catalase and acetylcholinesterase films (2). The activity of surface-spread trypsin was dependent on the surface concentration of the enzyme (3).

Estimation of catalytic activity of surface-spread enzymes presents unique difficulties, because the fraction of the enzyme that escapes into the bulk phase must first be removed. Previously (4), an apparatus and technique for studying monomolecular films were reported; the subphase can be exchanged without disturbing the film, thereby removing any dissolved contaminants in the bulk phase. Data were presented to demonstrate the utility of this system for studying protein monolayers and its applicability to the study of ion-exchange properties of phospholipid monolayers.

In this study, the subphase exchange technique was used to investigate the enzymatic behavior of surface-spread pig heart mitochondrial malate

dehydrogenase. Mitochondrial malate dehydrogenase, an enzyme of the tricarboxylic acid cycle, catalyzes the oxidation of L-malate to oxalacetate (5). This enzyme requires relatively drastic isolation procedures, including an acetone-dried powder preparation of tissues and an ethanol fractionation (6). The difficulty in its isolation suggests that the enzyme is firmly bound to the mitochondrial framework. Aqueous solutions of mitochondrial malate dehydrogenase are quite unstable, and most of the catalytic activity is lost on standing for a few hours at room temperature (7). This instability of the enzyme is associated with the removal of the enzyme from its bound state, which also suggests that it is situated at a biological interface.

A previous article (4) discussed film studies of pig heart mitochondrial malate dehydrogenase at an air–water interface with and without subphase exchange. Malate dehydrogenase forms stable films, which give reproducible surface pressure–surface area (π–A) curves. Films of malate dehydrogenase that were formed using different amounts of this enzyme were studied after the buffered subphase was changed with a buffer solution of the same composition. The exchange of this subphase had no effect on the π–A plots of any malate dehydrogenase film studied.

The purposes of this phase of the study were to investigate the applicability of the subphase exchange technique for studying the catalytic activity of surface-spread malate dehydrogenase and to determine the effect of surface pressure on catalytic activity.

EXPERIMENTAL

Materials—Malate dehydrogenase[1] (porcine heart) was supplied as a suspension in 70% saturated ammonium sulfate solution. Oxalacetic acid[2] (*cis*-enol) and nicotinamide adenine dinucleotide, reduced[1] (NADH), 90%, were used to measure the activity of the enzyme. The inorganic chemicals used, all reagent grade, were checked for surface impurities. The water used was first deionized by passing distilled water through a mixed resin bed of a demineralizer[3] and then distilled twice from an all-glass still. All buffer solutions were passed through a column of activated charcoal.

Surface tension measurements were made by the Wilhelmy plate method (8), using a thin roughened platinum plate suspended from a torsion balance. The film balance[4] consisted of a 500-ml capacity Langmuir-type polytef[5] trough equipped with a polytef movable barrier and two ribbon screw polytef stirrers running along the two long edges of the trough. The stirring system was designed so that the motion at reasonable speeds did not disturb the film. For all experiments, the temperature of the trough was maintained at 27 ± 2° by circulating water from a bath in a jacket surrounding the trough.

Monolayer Methods—The apparatus and procedure for subphase exchange and for determining π–A isotherms for protein monolayers spread at the air–water interface were described previously (4). The π–A isotherms of proteins were obtained by film compression after the protein was spread by the Trurnit (9) method. Compression was initiated 25 min after the protein was spread, which allowed enough time for equilibration and subphase exchange. In the exchange process, 900 ml of subphase was exchanged (trough capacity = 500 ml) with a phosphate buffer solution of the same composition as the subphase.

Spreading solutions of malate dehydrogenase were prepared by diluting a suspension of malate dehydrogenase with sufficient 0.1 *M* phosphate buffer (pH 7.4) to yield a concentration of 1.36 mg/ml. The subphase in the surface balance trough also consisted of 0.1 *M* phosphate buffer (pH 7.4). The pH value of 7.4 was chosen because the highest activity of malate dehydrogenase occurs in the physiological range of pH 7.3–7.5 (10). Different volumes of spreading solution of the same concentration of enzyme were delivered to spread films of different protein surface concentrations. For each experiment, a fresh spreading solution of malate dehydrogenase was prepared.

To determine the amount of enzyme adsorbed at the air–water interface after subphase exchange, films were removed by negative pressure using a narrow glass tube and then analyzed for protein content by a modification of the Lowry (11) technique.

Enzyme Activity Methods—For enzyme activity studies, a fresh solution of oxalacetic acid was prepared for each experiment by dissolving 50 mg of oxalacetic acid in 50 ml of pH 7.4 phosphate buffer and adjusting to pH 7.2–7.4. The solution was stored at 0°. Solutions of NADH were prepared by dissolving 112 mg of NADH in 50 ml of the pH 7.4 phosphate buffer immediately before use. This solution was never frozen.

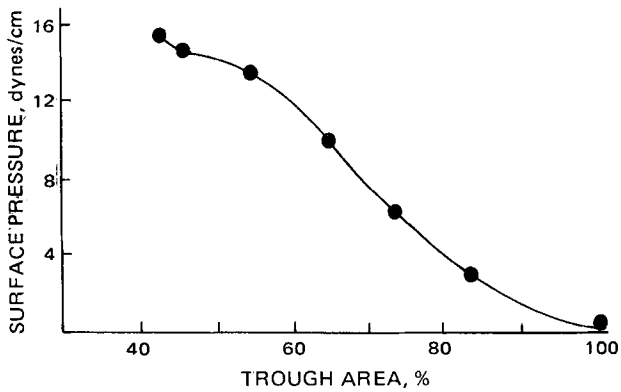

Figure 1—*The π–A curves of mitochondrial malate dehydrogenase films of 0.081 mg of enzyme spread on pH 7.4 phosphate buffer and the subphase was exchanged with 900 ml of buffered solution.*

The malate dehydrogenase film was first spread by the method described previously. Five minutes after the protein had been spread, the subphase exchange was started and continued for 15 min. In the process, 900 ml of the subphase was exchanged. After the exchange, the polytef barrier of the surface balance was moved to a position that gave the desired surface pressure at which the activity of the enzyme was to be measured.

Following compression, 40 ml of the NADH solution was injected under the film with two glass syringes attached to stainless steel needles previously placed at the midline of the trough 10 cm from each edge in the trough. Two injections were used instead of one to obtain a quicker mixing of the injected solution with the subphase. Injections were made at a slow, steady rate to avoid any disturbance of the film. The injection of air bubbles was also avoided.

Soon after injection of the NADH solution, the stirrers were started. The stirrer speed, at which there was no disturbance at the surface, was kept constant for all experiments. Seven minutes after the injection of NADH solution, oxalacetic acid solution was injected through the two syringes to initiate the reaction. The time at which the contents of the first syringe of oxalacetic acid solution was injected was taken as time zero for the reaction.

As soon as the oxalacetic acid solution was injected, a 4-ml sample of the subphase was pulled through one needle. Additional 4-ml samples were then pulled at specific intervals for UV analysis[6] at 340 nm. The UV absorbance of the subphase solution was independent of the part of the trough from which the sample was withdrawn. Throughout the experiment, the needles remained in place and the stirring was continued. The activity of the enzyme was measured spectrophotometrically by virtue of disappearance of NADH based on the characteristic absorption of NADH at 340 nm.

The slope of the absorbance–time curve, representing the rate of enzyme-induced oxidation of NADH to NAD, was used as a measure of malate dehydrogenase activity. Plots of NADH absorbance *versus* time were linear, and results were reproducible. Slopes were calculated using a linear regression computer program.

RESULTS AND DISCUSSION

The malate dehydrogenase films were spread on pH 7.4 phosphate buffer, and the subphase was exchanged with a buffer solution of the same composition. In all experiments, malate dehydrogenase formed stable films which gave reproducible π–A curves.

Figure 1 shows the π–A curve of 0.081 mg of malate dehydrogenase spread on buffer solution with the subphase exchanged. The π–A plot shows an inflection point at 15 dynes/cm. Beyond the inflection point, *i.e.*, at smaller surface areas, the film became less compressible and also less reproducible. This concentration of protein was chosen because it allowed observation of the catalytic activity of the enzyme film essentially from the gaseous state up to the point where the film became unstable, using the same trough without changing the amount of protein spread. The concentration of malate dehydrogenase at the surface must be

[1] P. L. Biochemicals, Milwaukee, Wis.
[2] Schwarz/Mann, Orangeburg, N.Y.
[3] Bantam.
[4] Frater Instrument Co., Corona, N.Y.
[5] Teflon (du Pont).
[6] Beckman DB spectrophotometer, Beckman Instruments, Fullerton, Calif.

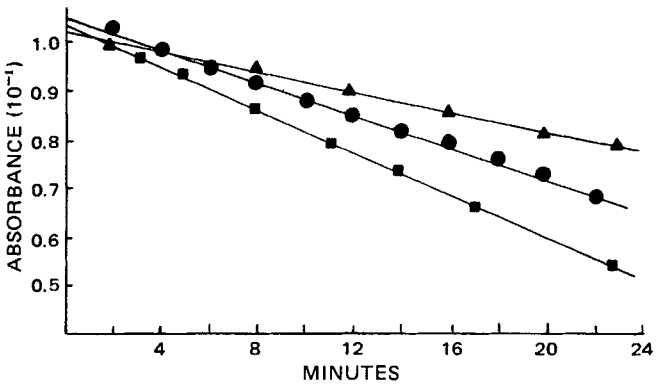

Figure 2—*Catalytic activity plots of surface-spread mitochondrial malate dehydrogenase (absorbance of NADH at 340 nm* versus *time) at full trough area. Key: ▲, 0.04 mg; ●, 0.06 mg; and ■, 0.08 mg.*

constant because the π–A plots using different concentrations of this protein are parallel but not superimposable (4). The π–A curves of higher malate dehydrogenase concentration films are condensed relative to those of lower concentrations.

Figure 2 shows some catalytic activity data of the surface-spread malate dehydrogenase. The plots of NADH absorbance *versus* time were linear and reproducible. The slopes of these plots were taken as a measure of malate dehydrogenase activity. The reactions were followed for about 20 min.

To investigate the effect of surface pressure on enzyme activity, compression of the enzyme film was necessary. The movement of the polytef barrier to compress the film resulted in a proportional decrease in area available to the enzyme at the surface. Since the reaction was carried out under zero-order saturated conditions, the available surface area of the enzyme at any given surface pressure should not have affected the reaction rate. To be certain that this was the case, different amounts of malate dehydrogenase were spread at the surface and the catalytic activity was studied at different available areas of the surface.

Figure 2 and Table I show the catalytic activity of different amounts of protein. Since the surface pressure of all films in this phase of the study was about zero, all of these films were gaseous in nature. First, 0.08 mg of protein was applied to the surface at full trough area and the enzyme activity of this film was recorded. The catalytic activity of 0.04 mg of enzyme spread at full trough area was then observed. In another experiment, 0.04 mg of enzyme was spread at full trough area and the area was then reduced to one-half the original trough area. The catalytic activity of this system was then studied. Similarly, the catalytic activity of 0.06 mg of enzyme was studied at full trough area and then after reduction to three-quarters trough area.

Table I shows that the activity of 0.04 mg of the enzyme, both at full trough area and at one-half trough area, was one-half of the catalytic activity observed for 0.08 mg of the enzyme. Similarly, the activity of 0.06 mg of the enzyme was about three-quarters of the activity observed for 0.08 mg of the enzyme both at full and three-quarters trough areas. These results indicate that the surface area available to the enzyme did not affect the catalytic activity when 0.08 mg or less of protein was spread as long as the surface pressure remained constant, *i.e.*, no conformational changes occurred. Thus, any changes of enzyme activity observed with compression of the film can be safely attributed to the change in surface pressure, *i.e.*, the conformational changes of the enzyme molecules associated with the change in surface pressure.

The orientation of the side chains of the surface-spread protein in relation to the surface may vary with the surface pressure (2). At film pressures close to zero, the side chains may lie flat in the surface. If the pressure is increased, the polar groups would be oriented into the aqueous phase while the nonpolar groups would be oriented toward the air phase. Therefore, it should be possible to vary the orientation of the protein molecules in a film by applying surface pressures of varying magnitudes.

Table I—Enzyme Activity of Malate Dehydrogenase Films

Amount of Enzyme Spread, mg	Surface Area Available to Enzyme	Enzyme Activity[a], Arbitrary Units
0.08	Full trough	2.2
0.06	Full trough	1.62
	Three-quarters trough	1.5
0.04	Full trough	0.98
	One-half trough	1.07

[a] Slope ($\times 10^2$) of absorbance of NADH at 340 nm *versus* time plot.

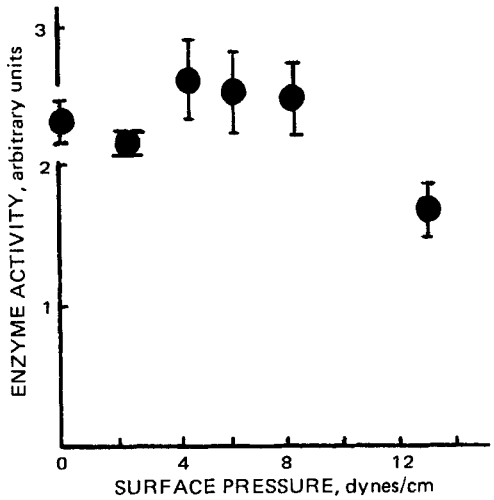

Figure 3—*Catalytic activity of mitochondrial malate dehydrogenase* versus *surface pressure plot of malate dehydrogenase films.*

In all subsequent experiments in this study, the same amount of enzyme, 0.081 mg, was spread each time. The concentration of the spreading solution was also kept constant.

Figure 3 shows the catalytic activity of surface-spread malate dehydrogenase at different surface pressures. A dependence of catalytic activity was observed with surface pressure. At intermediate film pressures, the activity of the enzyme was higher than at very low or very high surface pressures. The maximum activity was noted at 4.4 dynes/cm, and very small changes in catalytic activity were observed at surface pressures between 4 and 8 dynes/cm. Although not shown in Fig. 3, at a surface pressure of 18 dynes/cm, a point beyond the inflection point of malate dehydrogenase film (Fig. 1), a high bulk catalytic activity was observed.

The film studies of the enzyme showed that the π–A plots were not reproducible beyond 15 dynes/cm. This finding suggests that the enzyme film at this pressure becomes unstable and that some enzyme leaves the surface and diffuses to the bulk. This idea is borne out by the observation of significant catalytic activity in the subphase at a high surface pressure (18 dynes/cm).

These results show that the catalytic activity of malate dehydrogenase films is dependent on the surface pressure and, in turn, on the conformation of the enzyme molecules and suggest a mechanism by which the activity of enzymes may be regulated in membranes. A dependence of catalytic activity on surface pressure also was reported for surface-spread acetylcholinesterase and catalase (2). With acetylcholinesterase, maximum activity was attained at a surface pressure of 10 dynes/cm and decreased at both lower and higher pressures.

The results of the surface protein content analysis show that when 0.081 mg of enzyme was spread, 29.6% of the original protein spread was found at the surface after subphase exchange. This loss would be expected since, when a solution of protein is applied at the interface, some protein unfolds and anchors at the surface while the remainder escapes into the bulk in globular form. The amount of protein escaping to the bulk would be dependent on, among other factors, the amount of protein originally applied to the surface. When acetylcholinesterase was applied to the surface, 44–74% of the original protein applied anchored at the surface (2). In another study (3), 80% of the trypsin applied to the surface was lost to the subphase.

An amount of malate dehydrogenase equivalent to that present at the surface after exchange was used to compare enzyme activity in the bulk with that of surface-spread enzyme under similar conditions; *i.e.*, the final concentrations of the enzyme, oxalacetic acid, and NADH were kept the same as in the activity studies of the film. The enzyme activity at the surface was equal to 13% of the activity of the same amount of the enzyme in solution.

The loss of activity of enzyme adsorbed at an air–water interface has been shown by a number of workers. Catalase retained about 22% catalytic activity on adsorption at the air–water interface (12). The catalytic

activity was dependent on the surface pressure of a film of acetylcholinesterase, and the maximum activity observed was at a surface pressure of 10 dynes/cm (2). This activity was equal to 29% of the activity of the same amount of enzyme in solution. The fraction of the retained enzyme activity on adsorption at an air–water interface in the case of trypsin varied from 0.125 to 0.35, depending on the surface concentration of the enzyme (3).

This study demonstrated the effectiveness of the subphase exchange technique for investigating the catalytic activity of enzymes spread at an air–water interface, without interference of bulk reaction. Future reports will discuss the effects of various lipids on the catalytic activity of surface-spread malate dehydrogenase.

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Kinetics and Mechanisms of Hydrolysis of 1,4-Benzodiazepines III: Nitrazepam

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Abstract □ The hydrolysis of nitrazepam involves a two-step sequential mechanism. The intermediate is the ring-opened compound resulting from scission of the azomethine bond. The final products are glycine and 2-amino-5-nitrobenzophenone. Recyclization of the intermediate to nitrazepam occurs at pH values above the pKa of the intermediate, in the pH region where the amino group of the intermediate is not protonated. As opposed to chlordiazepoxide and oxazepam, the initial hydrolysis step occurs at the 4,5-bond, not at the 1,2-amide linkage. This difference is attributed to a preferential activation for hydrolysis of the azomethine linkage by the nitro group. The hydrolysis involves an uncatalyzed reaction, specific acid–base catalysis, and general acid–base catalysis for acetate and phosphate buffers.

Keyphrases □ 1,4-Benzodiazepines—nitrazepam, kinetics and mechanisms of hydrolysis □ Nitrazepam—kinetics and mechanisms of hydrolysis □ Hydrolysis—nitrazepam, kinetics and mechanisms □ Anticonvulsants—nitrazepam, kinetics and mechanisms of hydrolysis

Nitrazepam, 1,3-dihydro-7-nitro-5-phenyl-2*H*-1,4-benzodiazepin-2-one, belongs to the 1,4-benzodiazepine class of tranquilizing agents. This compound is centrally active (1), surpassing diazepam, a popular benzodiazepine, in anticonvulsant activity (2).

Preceding publications (3, 4) in this series reported the hydrolysis kinetics of 7-chloro-1,4-benzodiazepines. The present study concerned the hydrolysis of the 7-nitro analog, nitrazepam.

EXPERIMENTAL

Materials—Compound purity was verified by TLC. Nitrazepam[1] and 2-amino-5-nitrobenzophenone[1] were used as received.

Buffer Solutions—All buffer solutions were made with deionized, distilled water. They were adjusted to an ionic strength of 1.0 with sodium chloride, except in ionic strength effect studies where they were adjusted to ionic strengths other than 1. The pH at the temperature of the runs was measured with a digital pH meter[2] equipped with high temperature electrodes.

The buffer systems used were: pH 1.0–3.0, hydrochloric acid; pH 3.2–5.6, acetate; pH 6.4–7.4, phosphate; pH 7.9–9.5, borate; and pH 10.1–11, sodium hydroxide.

Kinetic Measurements—The kinetic studies of the hydrolysis of nitrazepam were followed spectrophotometrically. Details of the specific procedures were given previously (3) and are summarized as follows.

A stock solution of nitrazepam (6.3×10^{-3} *M*) in ethanol was diluted in an appropriate buffer to a final concentration for the kinetic run of 2.4×10^{-5} *M*. The reaction flasks (light protected) were maintained in a constant-temperature oil bath[3] controlled within 0.1° at selected temperatures between 70 and 85°. Samples were withdrawn at suitable time intervals and cooled to room temperature by quenching in an ice water bath. Visible and UV spectrophotometric measurements were made with a double-beam spectrophotometer[4].

Isolation and Identification—Thin-layer chromatograms were made on 20 × 20-cm glass plates coated with a 250-μm layer of silica gel GF_{254}. After lyophilization, the samples were dissolved in chloroform, spotted, and developed in a closed tank for approximately 1 hr with dioxane–benzene–hexane–7.4 *M* NH_4OH (45:50:70:5). The solvent front was allowed to travel 17 cm from the origin. After drying, the plates were visualized using a short wavelength UV lamp and then sprayed with ninhydrin aerosol (0.5%) to produce visible spots. Nitrazepam, 2-amino-5-nitrobenzophenone, and glycine were spotted as reference standards. An intermediate was isolated by preparative TLC and characterized by its IR[5], NMR[6], and high-resolution mass[7] spectra.

[1] Hoffmann–La Roche, Nutley, N.J.

[2] Model 701, Orion Research, Cambridge, Mass.

[3] Sargent model SW equipped with Sargent thermonitor model ST, R. L. Sargent Co., Dallas, Tex.

[4] Model 124, Coleman Instruments Division, Perkin-Elmer Corp., Maywood, Ill.

[5] Model IR8, Beckman Instruments, Fullerton, Calif.

[6] Model C60HL, Jeol, Medford, Mass.

[7] CEC-21-110, DuPont Instruments, Wilmington, Del.