Automated Enzyme Assays

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1. INTRODUCTION

The automated assay of enzyme activities is a major activity of clinical, food, environmental, research, and other analytical laboratories. The assay of enzymes in body fluids is the authors' area of expertise. Earlier reviews of laboratory automation are by Alpert (1) and Schwartz (2); the latter described the early days of continuous flow, i.e., Technicon AutoAnalyzer systems with brief mentions of alternate automated devices. Perez-Bendito and Silva (3) describe continuous flow as applied to rate methods, and Roodyn (4) in his book, describes the topic of automated enzyme analyzers up to about 1970 and gives detailed information on enzyme assays on the Technicon AutoAnalyzer including a FORTRAN program for data reduction; he also provides a detailed bibliography to about 1969. He discusses other early instruments for enzyme assays such as the Bausch and Lomb "Zymat 340," the Joyce Loebl "Enzymat," the LKB "Reaction Rate Analyzer," the AGA Medical "Autochemist," the Vickers "Multichannel 300," the Warner-Chilcott "Robot Chemist," the Beckman "Kintrac VII," the Gilford "Multiple Sample Absorbance Recorder," and the Smith Kline "Eskalab." The degree of automation of the AutoAnalyzer was superior to that of the other devices; this was surely the reason for the dominant position of Technicon equipment in clinical laboratories in the late 1960s and early 1970s. The addresses of the manufacturers of the above devices are given by Roodyn (4). This information is largely of historical interest and is included here for completeness. The above automated enzyme analyzers have nearly all disappeared from clinical chemistry laboratories.

The development and application of centrifugal analyzers, a major advance in automated enzyme assays, is described by Tiffany et al. (5). This device was the first to use computerized process control and data reduction. There are many reports in the literature that evaluate or compare specific instruments. Some
examples of such studies are those by Valcarcel and Luque de Castro (6) describing the Technicon RA-100, BMD Hitachi 705, and Beckman Astra-8 analyzers (7), the Abbott Spectrum (8, 9, 10), the DuPont ac(11, 12), the DuPont Dimension (13, 14), the Hitachi 704 (15, 16), the Hitachi 717 (17, 18), the Hitachi 736 and 737 (19, 20), the Kodak Ektachem 700 (21, 22, 23), and the Corning ACS 180 (24). Other reports make comparison of various instruments, e.g., the Hitachi 737 versus the Ektachem 700 (25), and the Baxter Paramax versus the Ektachem 700 (26). These studies deal with enzymes and other tests, comparability of the

Fig. 1. Part of the optical system of the Abbott Spectrum. The instrument uses a grating to isolate various wavelengths, and simultaneous measurements at multiple wavelengths are made with the diode array detector.
Fig. 2. Detail of the Baxter Paramax analyzer showing the handling of specimens. The instrument pierces the rubber septum to gain access to serum. The cuvets are provided as long spools for the continuous feeding into the instruments.
results with those from accepted methods for many analytes, quality control data, and other performance characteristics. Unfortunately, these reports become obsolete quickly owing to model changes, improvements of all types, reagent reformulations, and the like. Figures 1 to 7 show some representative details of contemporary automated enzyme analyzers with some special or unique features.

The distinction between "batch" and "discrete" analyzers is an important one. The AutoAnalyzer is a batch instrument; groups of specimens are analyzed together along with standards and quality control specimens or knowns. It is inefficient to analyze one specimen on the AutoAnalyzer owing to the required calibration with every run. Contemporary analyzers such as the DuPont aca and Kodak Ektachem are extremely stable, and only infrequent calibration is needed; these devices meet the needs of clinical laboratory analyses: any test at any time. Assaying specimens as they arrive in the laboratory provides better and more timely testing services rather than accumulating specimens and analyzing them in groups.

Our review covers the major developments in automated enzyme assays since about 1975. The well-documented advantages of automation are several: reductions in imprecision, increased productivity of the staff, standardization of methods, faster assay of specimens and reporting of results, and fewer human blunders.
Fig. 4. Detail of the Beckman CX 4/5 sample probe sequence showing the aspiration of specimen, delivery to the cuvet, and washing of the probe. The washing system is designed to minimize carry-over of specimen.
Fig. 5. Detail of DuPont Dimension Analyzer. Cuvets are formed on the instrument from two bands of nylon film, and absorbance measurements are made on these cuvets.
Fig. 6. Detail of the preprocessing device, the "Plus," for the DuPont aca analyzer. Separation and pretreatment steps are carried out here, and then the treated sample is added to an aca cup for analysis by the DuPont aca.

2. WHAT IS AUTOMATION?

As is discussed by others (6), "automation" is often poorly defined, and there are clearly many levels of automation (27). Modern, computer-controlled instruments require less-and-less human intervention. But is it desirable to have the instrument (or robot) replicate every human manipulation? The answer is a qualified yes, and we discuss here the rationale and precautions of various steps in the analytical chain and the safeguards that must be built into the instrument to maintain the integrity of the results.
Fig. 7. Detail of the Hitachi 717 analyzer. Reagents are stored in "R1" and "R2." Specimens and reagents are automatically pipetted into the "Reaction Disk" for incubation and photometry.
A comparison of some currently available automated enzyme analyzers is given in Table 1. Two caveats apply: The selection of instruments is based on those that have large peer groups in the College of American Pathologists (28) proficiency testing surveys, and continuing updates of equipment makes it highly likely that some of the instruments will have features not shown in the table.

3. SOLUTION-BASED, ABSORPTION SPECTROPHOTOMETRIC METHODS

Ultraviolet and visible absorption spectrophotometry are still the most widely used techniques for enzyme assays in aqueous systems. Nephelometry or turbidimetry are used for a few special situations, e.g., assay of serum or urine amylase with a starch solution as the substrate where the clearing of the solution is measured, or similarly, the assay of serum lipase where the clearing of an olive oil emulsion is followed. Table 2 shows the current list of clinically important enzymes and their substrates. Automated analyzers rely largely on “self-indicating” substrates such as NAD(P)H, p-nitrophenylphosphate, phenolphthalein monophosphate, thymolphthalein monophosphate, derivatives of 4-nitrophenyl maltopentaoside, and so on. These instruments generally do not permit a separation step to complete the analysis; the reaction vessel is also the cuvet or measuring container. The reaction chemistries may be quite complex; however, the specimen handling and spectrophotometry must be adapted to the mechanical requirements of the instrument. Microwell readers can serve nicely to measure enzyme activities simultaneously in 96 wells (29). Flow-injection analysis of enzyme is a variant of continuous flow spectrophotometry and has been applied to the assay of enzymes (30).

3.1. Specimen Identification

Identification is a major problem in clinical laboratories, and serious untoward events can occur with misidentified specimens. Unambiguous identification is possible today with bar-coding and similar machine-labeling techniques (31). The model discussed here is for testing patient-derived materials in a clinical laboratory; the model can, of course, be extended to other applications. A machine-readable label on every specimen is the contemporary standard of modern equipment. Keying in identifiers to an instrument is less desirable owing to the inevitable human errors. In our experience with bar-code readers, they “read” the label correctly or don’t work at all. The topic, automated specimen identifications, is described in more detail in Section 8.2 here (32).

3.2. Specimen Preparation

Preparation for analysis ranges from a mundane step such as centrifugation to sophisticated procedures like pretreatment by chromatography, extraction,
reaction with antibodies, and so on. The DuPont aca (see Table 1) uses some reagent packs that incorporate pretreatment columns in the packs, e.g., those for the assay of the CK-MB isoenzyme of creatine kinase (CK, EC 2.7.3.2). A pretreatment module for the aca, the aca plus, provides additional capabilities to carry out separation and isolation steps so that the treated specimens can then be analyzed in the usual way on the aca.

The Kodak Ektachem 250 uses reactions on thin films and a "radial wash" technique as a pre-analysis step for certain analytes. The Baxter Stratus instrument uses a similar strategy. The Corning ACS 180 employs a double-antibody technique to measure CK-MB and a fully automated separation step using antibody coated on magnetic particles; nothing is required of the operator except loading the reagents and bar-coded specimens into sampling wheels.

Removing the stopper from a tube of blood can be eliminated—and the occasional aerosol that is produced during opening—by piercing the stopper's rubber septum with the probe as is done on the Baxter Paramax or as has been suggested by Columbus and Palmer (33) in a special blood collection device that is centrifuged axially and then sampled through a port in the plastic tube. Others (34) have devised a plastic cone that is inserted into the rubber stopper that serves as an opening to the specimen and as a guide for the sampling probe. Safety concerns in the handling of potentially infectious specimens is stimulating more development in this area of automation.

3.3. Specimen Integrity

Unattended assays have unique requirements that manual techniques do not. On-instrument stability is an issue, particularly if the environment of the instrument is hotter than the laboratory bench, the typical case. For example, CK is unstable at 37°C, is light sensitive, and is easily oxidized during storage; such concerns impact the validity of CK assays (35). Specimen evaporation can be a serious problem, particularly for small specimens held in containers with large, exposed surfaces for prolonged periods of time (36, 37). Evaporation lids are desirable, but they cannot always be used owing to instrument constraints on the specimen probe.

3.4. Pipeting Specimens

Specimen sampling has special needs in automated instruments. The analyzer must detect an incomplete or "short" specimen, an air bubble, and any solid material such as a fibrin clot in a serum specimen or the silicon separator gel used in many blood collection tubes; failures of any of these invalidates the results, and the operator must be alerted. Use of anticoagulants is common in hospitalized patients, and therefore delayed clotting is a major problem in clinical laboratories. The instrument must sense when excessive force is needed to aspirate the specimen. Because it is impossible for the analyst to monitor the pipeting of specimens, these checks for possible failures must be in place, particularly with the typical 2μL to 20μL specimens in common use. The metering of tiny specimens is an art that is beyond the discussion here.
<table>
<thead>
<tr>
<th></th>
<th>Abbott Spectrum</th>
<th>Baxter Paramax</th>
<th>Beckman CX7</th>
<th>BMD Hitachi</th>
<th>DuPont Dimension AR</th>
<th>Kodak Ektachem 700 XR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Minimum sample vol., µL</td>
<td>1.25-10</td>
<td>5-20</td>
<td>5</td>
<td>1</td>
<td>14-40</td>
<td>11</td>
</tr>
<tr>
<td>Minimum total vol., µL</td>
<td>50</td>
<td>50</td>
<td>50</td>
<td>100</td>
<td>44-70</td>
<td>50</td>
</tr>
<tr>
<td>Closed-container sampling</td>
<td>N</td>
<td>Y</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>Cuvet type</td>
<td>Disposeable</td>
<td>Disposeable</td>
<td>Glass</td>
<td>Semi-disposable</td>
<td>Disposeable</td>
<td>Dry slides</td>
</tr>
<tr>
<td>Auto re assay</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
</tr>
<tr>
<td>Open channels</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
</tr>
<tr>
<td>Wash water usage</td>
<td>3 L/H</td>
<td>1 L/4500 tests</td>
<td>7 L/h</td>
<td>20 L/h</td>
<td>2 L/h</td>
<td>None</td>
</tr>
<tr>
<td>Wastestream</td>
<td>Cups, cuvets, wash</td>
<td>Wash, cuvets</td>
<td>Cups, wash</td>
<td>Cups, wash, cuvets</td>
<td>Cuvets, cups, wash</td>
<td>Slides, pipette tips</td>
</tr>
<tr>
<td>Liquid-sensing probe</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>Air-sensing probe</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>Clot-sensing probe</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>Optics</td>
<td>Grating</td>
<td>Filters</td>
<td>Grating</td>
<td>Filters</td>
<td>Filters</td>
<td>Filters</td>
</tr>
<tr>
<td>Time to perform one test (min)</td>
<td>10</td>
<td>10</td>
<td>8</td>
<td>12.5</td>
<td>3-7</td>
<td>7</td>
</tr>
<tr>
<td>Enzyme assays/hr</td>
<td>200</td>
<td>700</td>
<td>225</td>
<td>600</td>
<td>800</td>
<td>250</td>
</tr>
<tr>
<td>Calibration stability</td>
<td>Days</td>
<td>Weeks</td>
<td>Days</td>
<td>Days</td>
<td>Weeks</td>
<td>Weeks</td>
</tr>
</tbody>
</table>
Note. All instruments had the capability to make measurements in the visible or ultraviolet region of the spectrum, all had temperature thermostating, all had the capability to interface to a host laboratory computer with bidirectional communications and to retransmit data, all had direct-tube sampling and bar-code identification of the specimen, all had reagent identification to the instrument, and usually with bar codes.

1 The minimum amount of specimen that is used for an enzyme test.
2 The minimum volume for one test PLUS the "dead" volume in the specimen container that represents unusable specimen.
3 The instrument opens the specimen container and samples the contents.
4 Cuvet types can be plastic and replaced after a single assay or plastic and can be reused. Glass (or quartz) cuvets are multiuse and are washed between specimens.
5 The instrument senses when a specimen is out of range and automatically, without operator intervention, reassays the material on a dilution or on a smaller sample or by some other means.
6 An "open channel" is one the user can configure for their own particular needs or tests.
7 Water volume used for washing the cuvets and/or probe or for diluting specimens or reagents.
8 All instruments have specimen tubes in the wastestream; the above are in addition to this.
9 A probe determines if there is liquid in a tube.
10 The probe can tell if an air bubble has been aspirated.
11 The probe can tell if excessive force is needed to aspirate a specimen.
12 The device used to isolate wavelengths in the spectrophotometer.
13 The total time from inserting a specimen into the instrument to the production of the result.
14 The maximum number of assays per hour if only enzymes were assayed.
15 The approximate time between required recalibration.
<table>
<thead>
<tr>
<th>Enzyme (Abbreviation, IUB Group, EC No.)</th>
<th>Substrate</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alpha-amylase (hydrolase, EC 3.2.1.1)</td>
<td>α-1,4 links in D-glucose polymers, e.g., polyglucose (G6, G7, etc.), many starches, glycogen, dextrins</td>
<td>Does not cleave β-1-4 links as occur in cellulose, α-1-6 links at branches of starches, maltose</td>
</tr>
<tr>
<td>Acetylcholinesterase (hydrolase, EC 3.1.1.7)</td>
<td>Acetylcholine acetyl thiocholine</td>
<td>Moderately specific</td>
</tr>
<tr>
<td>Pseudocholinesterase (ChE, hydrolase, EC 3.1.1.8)</td>
<td>Many aliphatic esters of choline</td>
<td>Choline is HOCH₂CH₂N(CH₃)₃⁺, a quaternary amine. The OH group can be esterified with many groups; esters are substrates. CLEAVES PHOSPHATE ESTERS.</td>
</tr>
<tr>
<td>Acid phosphatase (ACP, hydrolase, EC 3.1.3.2)</td>
<td>Hydrolyses many phosphate esters, e.g., pNPP, G-6-P, phenyl-P, β-glycerophosphate, phenolphthalein-P, thymolphthalein-P, naphthol-P</td>
<td>ALP has unusual pH optimum of about 9. Optimum pH varies with substrate.</td>
</tr>
<tr>
<td>Alkaline phosphatase (ALP, hydrolase, EC 3.1.3.1)</td>
<td>Hydrolyses same phosphate esters as ACP</td>
<td>A nonspecific hydrolase; also acts on angiotensin I bradykinin, met-enkephalin, leu-enkephalin</td>
</tr>
<tr>
<td>Angiotensin converting enzyme (ACE, hydrolase, EC 3.4.15.1)</td>
<td>Splits peptides at certain sites, e.g., between GLY and PHE, also hippuryvl-L-histidyl-L-leucine is substrate</td>
<td>Absolutely specific; only reacts with L-aspartate or L-glutamate. Enzyme is stereo-specific.</td>
</tr>
<tr>
<td>Aspartate aminotransferase (AST, transferase, EC 2.6.1.1)</td>
<td>L-aspartate + 2-oxoglutarate or L-glutamate + oxaloacetate</td>
<td>Absolutely specific. Only reacts with L-alanine or L-glutamate. Enzyme is stereo-specific.</td>
</tr>
<tr>
<td>Alanine aminotransferase (ALT, transferase, EC 2.6.1.2)</td>
<td>L-alanine + 2-oxoglutarate or L-glutamate + pyruvate</td>
<td>Highly specific</td>
</tr>
<tr>
<td>Aldolase (ALD, lyase, EC 4.1.2.13)</td>
<td>Fructose-1,6-di-P</td>
<td>Non-specific. Capable of oxidizing many substrates.</td>
</tr>
<tr>
<td>Ceruloplasmin (oxidoreductase, EC 1.16.3.1)</td>
<td>Many oxidizable substrates e.g., phenylenediamine</td>
<td>Highly specific</td>
</tr>
<tr>
<td>Creatine kinase (CK, transferase, EC 2.7.3.2)</td>
<td>Creatine + ATP or creatine − P + ADP</td>
<td>Non-specific but requires gamma-glutamyl group.</td>
</tr>
<tr>
<td>Gamma-glutamyl transferase (GGT, transferase, EC 2.3.2.2)</td>
<td>Transfers a gamma glutamyl group from many &quot;donor&quot; peptides to an &quot;acceptor&quot; peptide</td>
<td></td>
</tr>
</tbody>
</table>
TABLE 2 (Continued)

<table>
<thead>
<tr>
<th>Enzyme (Abbreviation, IUB Group, EC No.)</th>
<th>Substrate</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glutamate dehydrogenase (GDH, oxido-reductase, EC 1.4.1.3)</td>
<td>2-oxoglutarate + NH₂⁺ + NADH or glutamate + NAD</td>
<td>Moderately specific</td>
</tr>
<tr>
<td>Lactate dehydrogenase (LD, oxido-reductase, EC 1.1.1.27)</td>
<td>Pyruvate and other ketoacids + NADH. Also lactate and other α-hydroxy acids + NAD</td>
<td>Moderately specific</td>
</tr>
<tr>
<td>Hexokinase (HK, transferase, EC 2.7.1.1.)</td>
<td>ATP + D-glucose, ATP + D-fructose, ATP + D-mannose, or ATP + 2-deoxy-D-glucose</td>
<td>Moderately specific</td>
</tr>
<tr>
<td>Pyruvate kinase (PK, transferase, EC 2.7.1.40)</td>
<td>Phosphoenol pyruvate + ADP or pyruvate + ATP</td>
<td>Absolutely specific</td>
</tr>
</tbody>
</table>

3.5. Temperature Control

Temperature control to maintain the reaction solution within +/- 0.1°C of the desired temperature is required for enzyme assays. The rule-of-thumb is that the rate of enzyme-catalyzed reactions doubles for every 10°C or about 7% increase in the rate for every degree increase. Copeland et al. (38) described the effects of temperature on the activity of purified alkaline phosphatase (ALP, EC 3.1.3.1) from human liver, intestine, placenta, and porcine kidney. All the enzymes exhibited linear Arrhenius (log activity vs. temperature) relationships, and the activity at 30°C was about 1.2-times that at 25°C. The activity at 37°C was about 1.7-times that at 25°C. The choice of temperature depends on course of the assay. For clinical work, 30°C is a compromise owing to the instability of some enzymes at 37°C, but the latter has the advantage of giving faster rates (39).

The National Institutes of Standards and Technology [NIST, formerly the National Bureau of Standards (40)] sells a high-purity gallium standard in a Teflon and nylon container [Standard Reference Material (SRM) no. 19681 for use as a temperature standard. The melting point is 29.77°C and can be used to calibrate thermometers and other temperature-sensing devices.

In the past, 25°C was the commonly used temperature for enzyme assays; this temperature has the disadvantage of requiring cooling of the reaction chamber or cuvets and of course slower enzyme reaction rates as compared to 30°C or 37°C. A number of professional societies (e.g., International Federation of Clinical Chemistry (IFCC), National Committee for Clinical Laboratory Standards
(NCCLS)] have recommended that 37°C be used as the standard temperature for enzyme assays, and this setting is now widely used in automated analyzers.

### 3.6. Absorbance Accuracy

In cuvet-based analyzers, absorbance accuracy is checked readily with solutions of either $\text{K}_2\text{Cr}_2\text{O}_7$ or $(\text{NH}_4)_2\text{Co(SO}_4)_2$, both in dilute $\text{H}_2\text{SO}_4$. Solutions of $\text{KNO}_3$ are useful in the ultraviolet region (see Table 3). For assays using NAD(P)H, $\text{K}_2\text{Cr}_2\text{O}_7$ is particularly convenient; its peak absorbance wavelength, 350nm, is close to that of NAD(P)H, 340nm. $\text{K}_2\text{Cr}_2\text{O}_7$ in 5 mmol/L $\text{H}_2\text{SO}_4$ is stable for years in well-closed, hard-glass containers. Such a solution containing 0.9350 g/L $\text{K}_2\text{Cr}_2\text{O}_7$ has an absorbance of 10.0 at 350nm, and suitable dilutions can be prepared with 5 mmol/L $\text{H}_2\text{SO}_4$ to check absorbance accuracy at 350nm, to see if the instrument is giving a linear absorbance response with concentration, and to check for stray light with a solution having a nominal absorbance of about 3.0 A. NAD(P)H can be used to check for linearity and stray light, but it is unsuitable for checking absorbance accuracy owing to its variable water content. Other useful compounds to check linearity are: oxyhemoglobin, p-nitrophenol, cyanometemoglobin, and biuret-protein complex prepared with BSA or HSA (41). Glass filters that fit 1-cm cuvet holders are available from the NIST as SRM 9301 for checking absorbance accuracy.

Grating and prism spectrophotometers should recover the theoretical absorptivities shown in Table 3; interference-filter photometers typically produce lower values, and glass-filter photometers produce lower values still. As a general rule, the wider the band-pass of the filter, the lower the absorptivity. In any case, even filter photometers should show constant absorptivities with time. Because many automated enzyme assays do not use a standard but rely on the absorptivity of the chromophore to determine the activity, low values of the absorptivity owing to a wide band-pass filter will yield lower enzyme activities.

Instructions on the use of $\text{K}_2\text{Cr}_2\text{O}_7$ solutions for checking absorbance accuracy are given in Appendix 1.

### 3.7. Wavelength Accuracy

Wavelength accuracy, i.e., does the instrument’s setting agree with the actual wavelength of the light, affects the accuracy of spectrophotometric assays. The emission lines of mercury or deuterium permit very precise wavelength settings; less satisfactory but still usable are holmium oxide or didymium filters, e.g., NIST SRMs 2009, 2010, 2013, and 2014. The problem with most automated enzyme analyzers is that the above techniques cannot be used owing to the configuration of the optics and cuvets, inability to insert a filter in the measuring chambers, and so on. Korzun and Miller (42) proposed a methyl red solution measured at two pH values to judge if the wavelength setting is correct. Their method does not establish wavelength accuracy but can be used to monitor shifts in wavelength.
### Table 3
Reagents for Checking Absorbance Accuracy (179)

<table>
<thead>
<tr>
<th>Material</th>
<th>Wavelength, nm</th>
<th>Conc., g/L</th>
<th>a, L/g-cm</th>
<th>Note</th>
</tr>
</thead>
<tbody>
<tr>
<td>K$_2$Cr$_2$O$_7$ (294.12)</td>
<td>257</td>
<td>0.0500</td>
<td>14.38</td>
<td>In 5 mmol/L H$_2$SO$_4$</td>
</tr>
<tr>
<td></td>
<td>350</td>
<td>0.0500</td>
<td>10.72</td>
<td>In 5 mmol/L H$_2$SO$_4$</td>
</tr>
<tr>
<td></td>
<td>257</td>
<td>0.1000</td>
<td>14.45</td>
<td>In 5 mmol/L H$_2$SO$_4$</td>
</tr>
<tr>
<td></td>
<td>350</td>
<td>0.1000</td>
<td>10.74</td>
<td>In 5 mmol/L H$_2$SO$_4$</td>
</tr>
<tr>
<td>(NH$_4$)$_2$Co(SO$_4$)$_2$ (287.14)</td>
<td>400</td>
<td>10.538</td>
<td>0.3406</td>
<td>In 1% H$_2$SO$_4$ (10 mL conc. H$_2$SO$_4$/L)</td>
</tr>
<tr>
<td></td>
<td>450</td>
<td>10.538</td>
<td>2.1062</td>
<td>In 1% H$_2$SO$_4$ (10 mL conc. H$_2$SO$_4$/L)</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>10.538</td>
<td>4.4550</td>
<td>In 1% H$_2$SO$_4$ (10 mL conc. H$_2$SO$_4$/L)</td>
</tr>
<tr>
<td></td>
<td>510</td>
<td>10.538</td>
<td>4.7466</td>
<td>In 1% H$_2$SO$_4$ (10 mL conc. H$_2$SO$_4$/L)</td>
</tr>
<tr>
<td></td>
<td>550</td>
<td>10.538</td>
<td>2.1117</td>
<td>In 1% H$_2$SO$_4$ (10 mL conc. H$_2$SO$_4$/L)</td>
</tr>
<tr>
<td></td>
<td>600</td>
<td>10.538</td>
<td>0.3733</td>
<td>In 1% H$_2$SO$_4$ (10 mL conc. H$_2$SO$_4$/L)</td>
</tr>
<tr>
<td></td>
<td>400</td>
<td>21.105</td>
<td>0.3537</td>
<td>In 1% H$_2$SO$_4$ (10 mL conc. H$_2$SO$_4$/L)</td>
</tr>
<tr>
<td></td>
<td>450</td>
<td>21.105</td>
<td>2.0680</td>
<td>In 1% H$_2$SO$_4$ (10 mL conc. H$_2$SO$_4$/L)</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>21.105</td>
<td>4.4554</td>
<td>In 1% H$_2$SO$_4$ (10 mL conc. H$_2$SO$_4$/L)</td>
</tr>
<tr>
<td></td>
<td>510</td>
<td>21.105</td>
<td>4.7075</td>
<td>In 1% H$_2$SO$_4$ (10 mL conc. H$_2$SO$_4$/L)</td>
</tr>
<tr>
<td></td>
<td>550</td>
<td>21.105</td>
<td>1.9864</td>
<td>In 1% H$_2$SO$_4$ (10 mL conc. H$_2$SO$_4$/L)</td>
</tr>
<tr>
<td></td>
<td>600</td>
<td>21.105</td>
<td>0.2993</td>
<td>In 1% H$_2$SO$_4$ (10 mL conc. H$_2$SO$_4$/L)</td>
</tr>
<tr>
<td>KNO$_3$ (101.10)</td>
<td>302</td>
<td>11.70</td>
<td>0.0708</td>
<td>In H$_2$O</td>
</tr>
</tbody>
</table>

#### 3.8. Time Control

Control of the reaction monitoring time is important for accurate enzyme assays. Electronic time control is used almost universally, but a discussion of this is beyond the scope of our chapter.

#### 3.9. Cuvet Control

Disposable plastic or permanent glass cuvets are being used in automated enzyme analyzers. Plastic cuvets can be manufactured to close tolerances for the solution path length and be sufficiently transparent in the near-ultraviolet spectrum. A disadvantage is cost and disposal. Permanent glass cuvets have better dimensional stability, and quartz cuvets have excellent transparency in the ultraviolet region to about 200nm. Their disadvantage is the necessary flushing, washing, and drying after each specimen and the build-up of protein on the walls.
when serum specimens are analyzed. Periodic replacement is necessary even for “permanent” cuvets, a significant cost item. On balance, plastic cuvets are probably superior for enzyme assays. Some automated analyzers monitor the cleanliness of cuvets prior to each use, an advantage. Standard 1-cm cuvets are available from the NIST as SRM 932.

3.10. Control of Interferences

With permanent cuvet systems, carryover of reagents and (or) specimens can be a problem. For example, if the same cuvet is used for a lipase assay and a triglyceride substrate and is then used to measure triglycerides in serum, a potential problem of triglyceride carryover exists. Turbidity of any cause, e.g., lipemia, can cause serious errors owing to the high background absorbance and the small change in absorbance therein for a specimen with low enzyme activity. Many automated analyzers will flag starting absorbances that are too high. A detailed examination of instrument- and method-associated interferences owing to icterus, hemolysis, and turbidity is available in the monograph by Glick and Ryder (43).

Interferences that occur in enzyme assays are numerous. The list of enzyme inhibitors prepared by Zollner (44) is invaluable. The work by Young (45) details many effects on enzyme results (or absence of same) caused by drugs and in-vivo changes or chemical, in-vitro interferences.

Another issue with sampling is possible cross contamination of adjacent specimens or specimen carry-over. At least two options exist: The instrument must wash or clean the probe between specimens, a sometimes fallible procedure because large wash volumes must be used if a low enzyme activity specimen follows one with tremendous activity. Alternately, a new pipette tip or probe is used with each specimen as is done on the Kodak Ektachem 700 avoiding the problem of contamination but adding the cost of pipette tips.

If the same probe selects different reagents for successive assays, then probe washing is an issue as is possible contamination of the reagent by the probe. In cuvet chemistries, mixing of the reagent with the specimen and/or the reaction-triggering agent must produce a uniform solution.

Some examples of problems found in the assay of serum enzyme are probably quite general: Many enzymes are not substrate specific but give interfering reactions with analytes present in serum. A few instances of nonspecificity are given as follows.

3.10.1. KETO ACIDS

Keta acids, such as acetoacetic or β-hydroxybutyric, present in the blood of patients with diabetes mellitus or after a prolonged fast, interfere in alanine aminotransferase (ALT, EC 2.6.1.2) assays and will give falsely increased values. The rate-limiting reaction is:
Alanine + 2-oxoglutarate $\leftarrow$ ALT $\rightarrow$ L-glutamate + pyruvate.

The indicator reaction is

Pyruvate + NADH $\leftarrow$ LD $\rightarrow$ Lactate + NAD.

A reaction that interferes is

Keto acids + NADH $\leftarrow$ LD $\rightarrow$ NAD + products.

In the design of the ALT assay, the LD (EC 1.1.1.27) is usually allowed to "burn out" the keto acids before the rate measurements are made. LD reagent must be free of ALT activity, and a water-blank specimen will reveal reagent contamination. In general, the reagent enzymes must always be checked for contamination with the enzyme being measured or any other enzymes that might interfere.

3.10.2. AMMONIUM IONS

Ammonium ions interfere in the assay of aspartate aminotransferase (AST, EC 2.6.1.1). The rate-limiting reaction is

Aspartate + 2-oxoglutarate $\leftarrow$ AST $\rightarrow$ oxaloacetate + L-glutamate.

The indicator reaction uses MDH (EC 1.1.1.37) and is

Oxaloacetate + NADH $\leftarrow$ MDH $\rightarrow$ malate + NAD.

However, endogenous glutamate dehydrogenase (GDH, EC 1.4.1.3) will allow the following reaction to occur yielding falsely increased AST values:

$2$-oxoglutarate + NH$_4^+$ + NADH $\leftarrow$ GDH $\rightarrow$ glutamate + NAD.

Obviously, reagents containing NH$_4^+$ ions must be avoided.

3.10.3. GLYCEROL

Glycerol is present at low concentration in serum; occasionally, patients have hyperglyceridemia. Endogenous glycerol must be consumed before triacylglycerol lipase (EC 3.1.1.3) is measured to avoid falsely increased lipase values.

3.10.4. OXIDIZED ENZYMES

CK has thiol groups that are oxidized easily; this is most noticeable in specimens that have been stored. Oxidized CK can be restored with thiol-reducing agents, but this generally takes more time than what is allowed in the lag phase, and the
enzyme activity will be underestimated. This problem is even more acute in lyophilized products that are being used as calibrators or control materials. Grossly erroneous calibration can occur if the CK is not fully reduced, i.e., fully activated, prior to use.

3.10.5. Amino-Alcohol Buffers

Amino-alcohol buffers are used commonly for alkaline phosphatase (ALP, EC 3.1.3.1) assays. Some sources of these buffers are contaminated with a potent ALP inhibitor, an obvious interferant (46).

3.11. Photometric Accuracy

The accuracy needed in the measurement of absorbances is related inversely to the measuring interval. The DuPont aca uses a 17-sec measuring time for kinetic enzyme assays. Thus for a specimen with an AST activity of 20 U/L and measured using NADH in the indicator reaction, then the absorbance change in 17 sec is only 0.035 A units. A trivial error of 0.004 A introduces an 11% error. Clearly, highly accurate spectrophotometry is needed here and in other automated enzyme analyzers that use very short measuring intervals.

3.12. Extracting Enzyme Activities from Spectrophotometric Data

Data reduction of spectrophotometric readings must consider the extreme situations that are encountered with patients' specimens. The kinetics of enzyme-catalyzed reactions are described in the excellent volume by Bergmeyer and Gawehn (47). The discussion here assumes solution-based reactions occurring in cuvets; kinetic, dry-film reactions are described elsewhere in this review. The factors discussed here include the lag phase, finding the linear portion of the rate curve, substrate depletion, highly turbid specimens, and highly active specimens. An example for calculating enzyme activities from spectrophotometric data is in Appendix 2.

3.13.1. Lag Phase

The lag phase is the time for the enzymatic reaction to reach maximum velocity; the latter is the rate that is zero-order in substrate, and substrate-saturation kinetics are assumed. At the beginning of the reaction, changes in concentration of the substrate are assumed to be trivial, and the substrate is present in large excess. The lag phase depends on the specimens' activity and the complexity of the reaction scheme being used. For example, the determination of LD with the pyruvate-to-lactate reaction has little or no lag phase, even with serum specimens having low activity:

\[
\text{Pyruvate} + \text{NADH} \overset{\text{LD}}{\longrightarrow} \text{lactate} + \text{NAD.}
\]

We can infer that the mechanism of the above reaction is simple and that the intermediates form and decay at a rate that is greater than the overall rate.
A coupled reaction is used for the assay of AST:

\[
\text{Aspartate} + 2\text{-oxoglutarate} \rightleftharpoons \text{AST} \rightarrow \text{oxaloacetate} + L\text{-glutamate}
\]

\[
\text{Oxaloacetate} + \text{NADH} \rightleftharpoons \text{MDH} \rightarrow \text{malate} + \text{NAD}.
\]

To estimate the AST activity, the first reaction must be rate limiting, and for this to be so, the oxaloacetate must reach a fairly high steady-state concentration; the time for this to occur is the lag phase. Generally, a large excess of MDH is used so that the rate of the second reaction (above) is always much greater than that of the first. If the AST activity is extremely high, the MDH may be insufficient to obtain accurate results. More MDH can be used, but the reaction may then be too fast for the measuring device to provide meaningful enzyme data.

Bergmeyer (48) gives some estimates of the required enzyme activity in the indicator reactions. The AST reaction scheme can be restated as:

\[
k_1 \quad k_2 \quad A \rightarrow B \rightarrow C,
\]

where \(k_1\) and \(k_2\) are the rates of the first and second reactions. Table 4 illustrates the effect of the relative values of \(k_1\) and \(k_2\) on the overall rate. Obviously, \(k_2\) must be more than 100-times \(k_1\) for the assay to be useful. Increasing the indicator enzyme activity to give a \(k_2\) of 1000 is not necessary unless many highly active specimens must be analyzed with good accuracy. For many enzyme assays, the latter yields too few measurement points to give a satisfactory rate curve necessitating dilution and reassay.

A three-step reaction scheme is used for the assay of CK (49). The reactions are:

\[
\text{Creatine-P} + \text{ADP} \rightleftharpoons \text{CK} \rightarrow \text{creatinine} + \text{ATP}
\]

\[
\text{ATP} + \text{glucose} \rightleftharpoons \text{hexokinase} \rightarrow \text{ADP} + \text{glucose-6-phosphate}
\]

\[
\text{G-6-P} + \text{NAD(P)} \rightleftharpoons \text{G-6-PDH} \rightarrow \text{NAD(P)H} + 6\text{-P-gluconolactone}.
\]

### Table 4

<table>
<thead>
<tr>
<th>(k_1)</th>
<th>(k_2)</th>
<th>Overall Rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.3</td>
<td>&lt;0.2</td>
</tr>
<tr>
<td>1</td>
<td>1</td>
<td>0.46</td>
</tr>
<tr>
<td>1</td>
<td>10</td>
<td>0.74</td>
</tr>
<tr>
<td>1</td>
<td>100</td>
<td>0.96</td>
</tr>
<tr>
<td>1</td>
<td>1000</td>
<td>0.993</td>
</tr>
<tr>
<td>1</td>
<td>Infinite</td>
<td>1.00</td>
</tr>
</tbody>
</table>
The CK assay has a lag phase of several minutes for specimens with a normal CK activity even in the presence of a large excess of the activities of hexokinase (EC 2.7.1.1) and glucose-6-P-dehydrogenase (G-6-PDH, EC 1.1.1.49). A long lag phase for the CK assay is predictable based on the above discussion for a two-step assay. Specimens with increased CK activities generally have shorter lag phases.

3.13.2. SEARCH FOR THE LINEAR REACTION-RATE CURVES

Algorithms exist for curve searching; many use only two points at a fixed time after the start of the reaction, an unsatisfactory approach for accurate work. At a minimum, a curve-searching algorithm must be implemented to trap certain errors, to identify when the lag phase is over, to be certain that there is an adequate linear portion of the curve, and that the region of substrate exhaustion has not been reached. With highly active specimens, substrate exhaustion may occur during the lag phase, and the absolute absorbance can be used to trap such results. Highly turbid specimens with low or high enzyme activities and with high starting absorbances present special problems. Usually centrifugation to remove lipids or dilutions solves the problem. The change in absorbance ($\Delta A$) of adjacent points can be compared until the $\Delta A$s are constant within defined limits. At least six $\Delta A$s over a period of 1–6 min should be obtained; the linearity of the rate curve can be tested with a standard linear regression algorithm and tested for outliers with the standard deviation of the residuals, i.e., $S(x,y)$, that is the SD of the points' distance from the least-squares, straight-line region in the Y direction. The value of $|S(x,y)/mean\,\Delta A| \times 100$ should be less than 10%; a greater value indicates excessive scatter or noise about the line and (or) loss of linearity owing to substrate exhaustion and hence unsatisfactory results.

4. SOLUTION-BASED ENZYME ASSAY METHODS OTHER THAN ABSORBANCE

4.1. Fluorescence

The classic text by Udenfriend (50) initiated the surge in interest in the use of fluorescence by analytical and clinical chemists. This approach was of interest because of its potential for significant increases in analytical sensitivity. In fact, the fluorescence signal by the chromophore is 100 to 1000 times greater than the sensitivity of absorbance measurements. Because of this level of analytical sensitivity, fluorescence has been applied almost exclusively to the measurement of compounds at low concentrations.

Despite its advantage of increased analytical sensitivity, fluorescence measurements have drawbacks: concentration effects (the so-called inner filter effect), background effects owing to Rayleigh and Raman scattering, solvent effects (for example, interfering nonspecific fluorescence and quenching), and, most commonly, sample matrix effects such as light scattering, interfering fluorescence absorption, and photodecomposition. Nevertheless, successful applica-
tions of fluorescence measurements to automated enzyme assays have been described (51).

Instruments that measure fluorescence are termed either fluorometers or spectrofluorometers. The distinction between the two types is based on the approaches that are used to separate the excitation and emission light into monochromatic light. Fluorometers use interference or glass filters, and spectrofluorometers use gratings or prisms.

4.2. Bioluminescence and Chemiluminescence

These approaches differ from fluorescence and other luminescent techniques such as phosphorfluorescence in that the excitation event is caused by a chemical reaction rather than photolumination. There are a number of reviews that detail the development and applications of luminescent technology (52, 53, 54, 55, 56, 57, 58) and instrumentation (59, 60). Particularly outstanding is the recent detailed review by Stanley (61) where more than 90 luminometers (manual, automatic, microtiter plate, HPLC, LC, GLC, imaging, and others) from more than 60 sources are described.

Bioluminescence is a unique type of chemiluminescence found in biological systems; these reactions can be classified as either pyridine- or adenine-nucleotide linked systems or enzyme-substrate systems. In clinical enzymology, the most commonly used system is the firefly luciferin-luciferase system for the measurement of ATP:

$$\text{Mg}^{++} \quad \text{LH}_2 + \text{E} + \text{ATP} \rightarrow \text{E.LH}_2\text{-AMP} + \text{Products}$$

$$\text{E.LH}_2\text{-AMP} + \text{O}_2 \rightarrow \text{E} + \text{oxyluciferin} + \text{CO}_2 + \text{AMP} + \text{light},$$

where LH2 is firefly luciferin and E is firefly luciferase. As is evident from the above reaction, all enzymes and metabolites that participate in “ATP reactions” or that are linked to such reactions could be assayed by the luciferase bioluminescent assay, and indeed it has been applied to the direct measurement of adenylate kinase, pyruvate kinase, and hexokinase. Of greatest interest has been the application of the above reaction to the measurement of CK and the B-subunit activity of the CK-B isoenzymes (62, 63). The use of chemiluminescence has been limited severely by the general lack of a fully automated luminometer with a rate of specimen throughput comparable to that of existing major automated enzyme analyzers. One of the few luminometers that may provide the advantages of a fully automated system is the Auto-Clinilumat™ LB 952T/16 (64), which is capable of measuring chemiluminescence at up to 250 specimens per hour. The instrument can record chemiluminescence originating from solutions, coated tubes or beads, or magnetic particles. The emitted light is measured by a high-sensitivity, low-noise photomultiplier with wavelength range of 390nm–620nm and operated with an ultrafast photon counter. The algorithm to calculate the standard curve is a smoothed spline function following a logit-log or log-log transformation of the data.
4.3. Turbidimetry and Nephelometry

These techniques measure scattered light that is the result of light interacting with suspended particles in a solution matrix. Turbidimetry is the measurement of the decrease in the intensity of the incident light owing to phenomena such as reflectance, scattering, and absorption as it passes through a suspension of particles. In turbidity, measurements of the change in light intensity are made in a manner analogous to how absorbance measurements are made; that is, at 180° from the incident beam. Because of this similarity, turbidity measurements can be made on most automated enzyme analyzers. One of the main concerns with turbidity measurements is the signal-to-noise ratio, therefore it is critical that analyzers making such measurements have photometric systems with electro-optical noise of 0.0002 absorbance units or less.

Nephelometry measures light scattered toward a detector that is not in the direct path of the transmitted light. Instruments like the Beckman ARRAY™ (65) rate nephelometer, that is, a new generation of Beckman’s Immuno Chemistry System (ICS), measures the forward scatter at 70° to the incident beam to take advantage of the increased forward-scatter intensity resulting in greater analytical sensitivity. Light sources commonly used in nephelometry are xenon or quartz halogen lamps and lasers. The latter are particularly useful because of their high intensity and better-defined properties. The Behring Nephelometer Analyzer (66) uses a laser light source to measure light scattering at 13°–24° from the incident light at two time points after mixing of the assay components (67, 68).

Light-scattering measurements, whether turbidimetric or nephelometric, have been applied to immunoassays of specific proteins (69) such as immunoglobulins, transferrin, albumin, haptoglobin, C3/C4, and haptens such as theophylline, gentamicin, and tobramycin. Enzymes that have received any attention in this regard are amylase (70, 71) and lipase (72, 73, 74, 75, 76, 77). A difficulty with light-scattering assays for amylase and lipase is the restriction on the substrate concentration that must be low enough to allow some signal to reach the detector; generally, these concentrations are suboptimal for the enzyme assays (78).

4.4. Fluorescence Polarization

Fluorescence polarization (79) has been applied to the measurement of enzymes; however, these are generally mass assays rather than assays of enzyme activity.

5. NON-LIQUID REAGENT SYSTEMS

The terminology used to describe this technology is diverse enough to include such descriptions as “nonliquid” or “dry” reagent systems or “solid-phase” or “film” technology. In the context of this review, we will refer to the technology as
nonliquid reagent systems. However it is termed, these systems represent one of the more innovative analytical technologies.

Nonliquid reagent systems can be regarded as complete analytical packages and typically take the form of either thin pads or films. They are complete in the sense that all the reagents required are distributed in a dry form either throughout the supporting element, as in the case of the Seralyzer (80) or compartmentalized into specific layers as in the case of the Kodak Ektachem system reagent slides.

5.1. Advantages of Nonliquid Systems

Simplicity, convenience, and the ability to use small specimen volumes, a particular advantage in pediatric and geriatric populations, are among the advantages of these systems. Because of their “dry” form, nonliquid reagent systems provide a remarkably efficient approach to stabilizing labile components, such as coupling enzymes and, because of this, these systems have prolonged storage stability, even at room temperature. Such potential for extended storage further increases the convenience of these analytical devices. For example, the spreading layer of the Kodak Ektachem test slide can not only trap cells, crystals, and other particulate matter, but it can also separate low-molecular weight compounds from potential larger-molecular weight interferents, e.g., proteins. For some tests, but not enzymes, the solution reaching the reactive underlayers resembles a protein-free filtrate. Compounds that reflect light such as TiO₂ and BaSO₄ are usually incorporated into the spreading layer to hide or mask color or turbidity that may be present in patients’ specimens. The Reflotron (81) and the Ames Seralyzer nonliquid reagent systems use a film membrane to exclude blood cells that interfere with test procedures.

5.2. Evaluations of Nonliquid Systems

There are many reports that evaluate the performance of the nonliquid reagent systems; however, owing to proprietary constraints, there are relatively few articles that describe the actual preparation or detail the characteristics of these analytical devices. This veil of secrecy is particularly true of the Drichem (82) and Konica nonliquid reagent systems; information on these systems is confined almost exclusively to the patent literature. Very few clinical or performance evaluations have been reported. Therefore, these systems will not be dealt with in this overview. The OPUS (83) and the Stratus II (84) Immunoassay Systems are also nonliquid reagent systems that deal with the immunoassays of therapeutic drugs, fertility hormones, thyroid function, and other metabolites. The Stratus II can also be used to measure CK-MB as the protein rather than the enzyme. An excellent review by Chan (85) describes the hardware and performance of the OPUS, Stratus, and other such systems.

The principles and applications of nonliquid reagent systems have been reviewed (86, 87, 88). Particularly useful are the reviews by Zipp and Hornby (89) and by Campbell and Price (90).
The only nonliquid reagent system that appears in the College of American Pathologists surveys of laboratories is the Kodak Ektachem (91). Because of its widespread use, this system will be described in detail as being representative of nonliquid reagent systems. The Seralyzer and the Reflotron System are also nonliquid reagent systems that measure enzymes. Reviews have been published detailing specifics regarding the Seralyzer (92, 93, 94, 95, 96, 97) and the Reflotron (98, 99, 100).

5.3. The Ektachem Systems

The Ektachem systems (101, 102, 103, 104, 105) are high-throughput, random-access analyzers. Tests are performed on a batch of specimens, and the assays are selectable by using different reagent-impregnated slides. Each specimen can be assayed for a different number of tests. It is possible to obtain only one test per slide, and the used slides are discarded. Figure 8 illustrates the integration of the various modules of an Ektachem 700 analyzer. Cartridges, usually containing 50 slides, are kept in either one of two slide supply compartments. Magnesium salt pads in one compartment maintain the relative humidity at about 33% whereas desiccant packs maintain the relative humidity in the other compartment at about 15%. Amylase and lipase, both being measured with two-point rate techniques, use slides that are stored in the higher-humidity slide compartment whereas the slides for all the other enzymes are stored in the low-humidity slide compartment. Specimens are placed in sample cups or tubes that are covered with cross-cut, flexible plastic caps to prevent evaporation. Each specimen holder or quadrant contains disposable pipets (sample probe tips) mounted with each specimen that eliminates both the contamination of one sample by another within the probe and any carryover of one specimen into the specimen in the next sample cup. The sample probe tip is mounted automatically onto a single, positive-displacement piston in the metering tower that aspirates as much specimen volume as is required. For enzyme assays, volume requirements are either 10μL or 11μL. The piston operates in such a manner that an air space is created between the piston and the sample and, therefore, the piston does not touch the specimen. Although a piston operated on the principle of positive displacement may not always deliver exactly the same amount of serum, particularly because the viscosity may vary from individual to individual, it really is not necessary to deliver exact volumes because of the spreading layer concept and the arrangement of the optics in the Ektachem system.

5.3.1. Fluid Dynamics on the Ektachem

To initiate the assay process, the specimen is partially expelled from the piston and the 10μL or 11μL drop is touched to the spreading layer of the slide. Figure 9 illustrates the cross-section of a typical Ektachem slide. The thickness of the spreading layer ranges from 100μm to 300μm with void volumes of 60%-90%. Dimensions such as these provide a suitable structure that allows a rapid and uniform spreading of specimen before it reaches the reagent/indicator under-
Fig. 8. Layout of the Modules of an Ektachem 700 Analyzer showing the "rate incubator" for rate-reaction measurements, the "potentiometric incubator," and "colorimetric incubator." The latter two are not used for enzyme assays.
layers. Once applied to the surface of the spreading layer, the sample drop diffuses into the matrix (reagent and indicator layers) where it dissolves the components of the reaction to ultimately produce a chromogenic product that is measured by reflectance spectrophotometry (106). Some specific examples of Ektachem enzyme assays are shown in Fig. 10, and Table 5 summarizes some of the properties of all the enzyme assays currently available on the Ektachem systems.

5.3.2. SPECTROPHOTOMETRY ON THE EKTACHEM

After the specimen has been applied to the slide, a distributor arm moves the slide to the proper incubator: CM for the colorimetric and two-point rate enzyme tests (acid phosphatase, amylase, and lipase), PM for the potentiometric chemistries, and RT for the rate or kinetic incubator for the multiple-point rate enzyme chemistries. Temperature control within either the CM or RT incubator is maintained at 37 ± 0.1°C by contact of the slide with the rotating thermal mass of the incubator. The products forming in the slides in either the CM or RT incubator are monitored at what are termed read stations by separate reflectance densitometers or reflectometers. There are, however, differences on how such measurements are made. For the enzyme slides in the CM incubator, at selected
Fig. 10. Examples of enzyme assays on the Ektachem Analyzer showing the reagents in the various layers: (A) alanine aminotransferase, (B) alkaline phosphatase, and (C) creatine kinase.
<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Calibration Mode</th>
<th>Sample Volume (μL)</th>
<th>Wavelength (nm)</th>
<th>Substrate or Indicator</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acid phosphatase</td>
<td>Two-point rate</td>
<td>10</td>
<td>600</td>
<td>Fast red TR and azo dye</td>
</tr>
<tr>
<td>Alanine aminotransferase</td>
<td>Multiple-point rate</td>
<td>11</td>
<td>340</td>
<td>Alanine and NADH</td>
</tr>
<tr>
<td>Alkaline phosphatase</td>
<td>Multiple-point rate</td>
<td>11</td>
<td>400</td>
<td>p-Nitrophenyl phosphate/ p-nitrophenol</td>
</tr>
<tr>
<td>Amylase</td>
<td>Two-point rate</td>
<td>10</td>
<td>540</td>
<td>Dyed starch and dyed saccharides</td>
</tr>
<tr>
<td>Aspartate aminotransferase</td>
<td>Multiple-point rate</td>
<td>11</td>
<td>340</td>
<td>Aspartate and NADH</td>
</tr>
<tr>
<td>Cholinesterase</td>
<td>Multiple-point rate</td>
<td>11</td>
<td>400</td>
<td>Butyrylthiocholine and ferricyanide</td>
</tr>
<tr>
<td>Creatine kinase (total and MB)</td>
<td>Multiple-point rate</td>
<td>11</td>
<td>670</td>
<td>Creatine phosphate and leuco dye</td>
</tr>
<tr>
<td>Gamma-glutamyl transferase</td>
<td>Multiple-point rate</td>
<td>11</td>
<td>400</td>
<td>L-Gamma-p-nitroanilide and p-nitroaniline</td>
</tr>
<tr>
<td>Lactate dehydrogenase</td>
<td>Multiple-point rate</td>
<td>11</td>
<td>340</td>
<td>Pyruvate and NADH</td>
</tr>
<tr>
<td>Lipase</td>
<td>Two-point rate</td>
<td>10</td>
<td>540</td>
<td>1-Oleoyl-2,3-diacylglycerol and leuco dye</td>
</tr>
</tbody>
</table>
times, the slides are removed from the CM incubator and moved to the read station (also heated at 37°C) of the corresponding reflectometer to make the necessary reflectance reading. For the multipoint-rate enzyme chemistries (see Table 5), up to 54 readings, approximately one reading every 5-7 sec, are made at the appropriate wavelength during the 5-min time period when the slide is still in the RT incubator. A high degree of precision in the readings is attainable; the standard deviation of the tolerance around the reflectometer is 0.0004 reflectance transmission units.

Figure 11 is a schematic of the Ektachem reflectometer; it consists of a lamp and a series of optics, and the light uniformly illuminates the slide at a 45° angle (illumination optics). The diffuse reflected light is collected (detector optics) by a fiber-optic bundle (photodiode) located at a 90° angle to the plane of the underside of the slide (Fig. 11). Because reflected light is only collected from a very small, well-defined area of about 3 mm in the center of the slide, the process is essentially volume independent; applying more specimen to the slide merely increases the size of the chromogenic product spot. A filter wheel containing narrow-band interference filters of different wavelengths is located in the path of the reflected light. It is important to note that reflectance measurements are made on the surface opposite to the specimen application side. This arrangement avoids the need to read the product of the reaction through the overlaying spreading layer (Fig. 9) that may contain potentially interfering compounds. Finally, the output of the photodiode is digitized and then passed on to the main computer for further processing.

5.3.3. PROCESSING OF ENZYME DATA ON THE EKTACHEM

The mathematical relationship of incident and reflected light is complicated by such phenomena as diffuse reflectance, ordinary transmittance, and specular reflectance of hemispherically distributed incident light. Based on an analogy to transmittance density in wet chemistry methods, a mathematical relationship that takes into account all such phenomena is as follows:

\[ D_r = \log \left( \frac{\text{incident light}}{\text{reflected light}} \right) \]
\[ = \log \left( \frac{R_{\text{white}} - R_{\text{dark}}}{(R_{\text{test}} - R_{\text{dark}})} \right) \]

where \( D_r \) is the reflective density of the slide or film element and is analogous to absorbance in transmission spectroscopy; \( R_{\text{test}} \) is the intensity of the light reflected from the test slide, \( R_{\text{white}} \) and \( R_{\text{dark}} \) refer to the reflectance of the analyzer’s internal standards that span the range of potential reflectance measurements. Note that \( R_{\text{white}} \) is the intensity of reflected light read by the detector with a standard reflector (for example, a barium sulfate surface), and \( R_{\text{dark}} \) corrects for nonlinear effects caused by “flare light” and may be used to correct for other factors, if any, causing nonlinearity. The reflective density, \( D_r \), can be transformed into transmittance density, \( D_t \), which is linearly related to concentration or activity, using the transformation described by Williams and Clapper (107):
Fig. 11. Schematic of the Ektachem reflectometer. Specimen is added to the top, and reflectance measurements are made from the bottom of the slide.
$$D_t = 0.149 + 0.469D_r + (0.422/1 + 1.179e^{3.379D_r}).$$

Finally, the concentration of the analyte can be determined with:

$$C = B(D_t - D_b)$$

where \(C\) is the analyte concentration or enzyme activity, \(B\) is the reciprocal of the absorbance coefficient (a constant but analyte-dependent factor), and \(D_b\) is the blank reflectance density, that is, when \(C = 0\). Curme et al. (102), Morris et al. (108), and Campbell and Price (90) provide more specific details regarding the photometric transformation of reflectance density to transmittance density.

The process whereby the analyzer uses reflectance readings to determine enzyme activity can be generalized in the following steps:

1. Accumulation of either 54 or 2 reflectance readings depending on whether the reaction is a multiple- or two-point rate (see Table 5).
2. Performing a \(D_t\) to \(D_r\) transformation to linearize the data and also remove any potential optical interferences.
3. Removing any outlier readings (with the multiple-point rate enzymes) and calculating the first derivatives of the remaining raw responses to define the linear portion of the rate curve. Early readings prior to steady-state conditions (lag time) are not used in the calculations, and later readings that are beyond certain defined tolerances of constant velocity are also not used to calculate reaction rates. Two-point enzyme rates (acid phosphatase, amylase, and lipase) do not undergo such iterations, rather, the program simply uses the two readings taken at the defined times.
4. Application of an algorithm to determine the rate of change in reflectance. A least-squares regression algorithm is used with enzymes whose response curve approximates linearity. In the case of \(\gamma\)-glutamyltransferase, which does not yield a sufficiently linear response, the curve is fit with a linear combination of orthogonal polynomial expressions and the maximum rate is established. Figure 12 represents these previously mentioned steps. At this point, the instrument has generated a calculated rate for each specimen that must be converted into the recognizable units of enzyme activity, U/L, and this is accomplished using a calibration curve.

The calibration curve for enzymes is defined by the relationship (calibration model):

$$C = A_0 + A_1 G(\text{rate}) + A_2(\text{rate})^k,$$

where \(C\) is the enzyme activity, \(A_0, A_1, A_2\), and \(k\) are constants that define the curve and represent, respectively, the intercept, slope, and curvature; \(G\) is the transform, and the \(k\) exponent is equal to either 0 or 2 and is method dependent. For enzymes, \(k\) is equal to 2. The calibration curve is generated by the instrument.
Fig. 12. Steps employed in the processing of enzyme data: (A) accumulate reflectance readings, (B) Prepare first derivative trim of the raw responses, (C) calculate rate for linear-response enzyme curves, or calculate rate for nonlinear responding tests like γ-glutamyltransferase.
manufacturer by analyses of 100 to 200 patients' specimens, usually over five days. The testing of each specimen on an Ektachem analyzer (rate analyzer response) and by the reference method is performed concurrently. Once the calibration curve or model has been defined by the analysis of patients' specimens, the enzyme activities are defined for three calibrator fluids with stabilized enzymes that were also assayed during the five-day process; this aspect is termed SAV or Supplementary Assigned Value assignment. Calibration data that includes SAVs, splines (transforms that linearize the calibration system), the $k$ value (calibration model curvature term), and particularly for enzymes, information used in rate calculations (rate parameters) are provided to the user on diskettes. Subsequently, any rate arising from the assays of an unknown sample can be converted to activity using the stored calibration curve.

6. AUTOMATED ENZYME ASSAYS IN OTHER FIELDS

Enzyme assays in fields other than clinical chemistry generally are not as automated because there is typically not the same demand for throughput; there are notable exceptions in facilities that perform an extensive number of tests on animals.

6.1. Animal Testing

In veterinary medicine, enzymes used for diagnostic purposes are essentially the same as those used in the diagnosis of human diseases. There are, however,
significant differences in the reference intervals among the different species and, therefore, particular attention must be paid to determining, and using, the proper reference interval in order to make a valid diagnostic assessment. In the pharmaceutical industry, because a great deal of the testing is related to toxicity studies, the most commonly measured enzymes are those that are indicators of liver, heart, and kidney damage, and these include not only the transferases and $\gamma$-glutamyltransferase but also sorbitol dehydrogenase (EC 1.1.1.14) (109). Measurement of urinary enzymes (110, 111, 112) as indicators of kidney function in toxicological studies is also common in the pharmaceutical industry.

6.2. Environmental Testing

In environmental testing, direct measurements of enzymes is not common but rather by-products of bacterial growth or contamination such as nitrate (113) and sulfite (114, 115) are measured using enzymatic procedures. Lead poisoning in smelter workers and children has become a serious societal concern and direct measurement of enzymes such as alanine dehydrogenase (EC 1.4.1.1), coproporphyrinogen oxidase (EC 1.3.3.3), and ferrochelatase (EC 4.99.1.1) has been suggested as predictors of lead toxicity (116, 117, 118, 119). Cholinesterase (EC 3.1.1.8) is valuable as a marker of hepatotoxicity and is used extensively to monitor workers involved with organophosphates (120, 121, 122). Measurement of enzymes released from lysosomes (exoenzymes) has been suggested as a measure of pollutant toxicity, i.e., organic matter degradation and nutrient regeneration in aquatic environments (123, 124). Measurement of $\alpha$-amylase in certain cereals such as wheat is used to establish the fitness of the cereal for human or animal consumption (125). Increased activity of $\alpha$-amylase in the cereal is indicative that the cereal has entered its "sprouting" phase and is no longer suitable for human use.

7. QUALITY CONTROL AND STANDARDIZATION OF ENZYME TESTS

7.1. Quality Control

Internal quality control is basically process control of day-to-day work. Control materials should mimic patients’ specimens, and the frequency of control assays is dependent on the stability of the instrument and reagents. With contemporary instrumentation, much less frequent assaying of quality control materials suffices, e.g., once per 8-hr shift is typically quite satisfactory and results in considerable savings. Quality control is a very large topic that has been discussed extensively elsewhere, especially by Westgard and Barry (126) and Lott and Patel (127). A new issue is the use of "medical-needs criteria" in setting control limits as are typically done with Levey-Jennings charts. With today’s much more precise instrumentation, e.g., the DuPont aca and Kodak Ektachem, limits based on past performance in routine testing are usually inappropriately narrow leading
to unnecessary reassays, recalibration, and other steps that serve neither the patient, the physician, or the laboratory. The quality control of enzyme tests in particular must consider how the results are used. Physicians generally disregard small to moderate changes in enzyme values, particularly if the values are abnormal (128). In fact, if the values are highly abnormal, say 10-times the upper reference or normal limit, then a threefold-upper normal change in the value is generally disregarded. Such medical decision making should be translated to routine process control: Extreme precision is not needed for enzyme tests, and within-laboratory CVs of <10% for the common enzyme tests is generally satisfactory.

7.2. Standardization: Now and Proposed

Currently, measurement of enzymes is based on catalytic-activity measurements, and these depend on the experimental conditions under which the enzyme is measured. It is often difficult to compare enzyme results from different laboratories or to those reported in the literature. Efforts to improve interlaboratory comparability in enzyme measurements (129, 130, 131, 132, 133, 134) have concentrated on two aspects: standardization of methodology and preparation of reference materials (135).

There have been considerable efforts over the last 10 years or so by national, particularly the Scandinavian Committee on Enzymes of the Scandinavian Society for Clinical Chemistry (136), and internationally by the IFCC to institute reference methods for many of the clinically important enzymes (137). The IFCC, through its Expert Panel on Enzymes, played a pivotal role by recommending reliable methods and clearly documenting the evaluations and recommendations being proposed. IFCC-recommended reference methods have been published for aspartate aminotransferase (138), alanine aminotransferase (139), y-glutamyltransferase (140), alkaline phosphatase (141), and creatine kinase (142) with work just about complete on a-amylase and lactate dehydrogenase.

In many cases, standardization of enzyme methodology has significantly reduced but not eliminated interlaboratory variability (133, 134, 143). The use of recommended, reliable reference methods has hastened the elimination of unsatisfactory methods and focused the attention on the use of methods that, if not exactly like the reference method, are based on the same methodological principles. Results from external quality control schemes clearly demonstrate that it is only through the use of essentially the same recommended methods (standardization of methodology) and the availability of an enzyme reference material (ERM) that laboratories can improve their performance (133). Any system for the standardization of the catalytic activity of enzymes not only requires available reference methodology but also stable and well-characterized enzyme reference materials (135) because standardization of enzyme methodology has not been achieved, and there is some question as to whether or not it will ever be attained. National recommendations are strongly supported in some areas at the
expense of international consensus. New developments in methodology and instrumentation in clinical enzymology will continue to be introduced, and small changes by the manufacturer in either the quality or concentrations of the reagents in a kit originally formulated as following the reference methodology criteria may lead to perhaps small, imperceptible changes in the reference method that may, over time, change its analytical performance. Some (135) envisioned enzyme reference materials, particularly in relation to the IFCC reference methods, as being used to: (1) maintain the IFCC methods; (2) transfer the IFCC methods to clinical laboratories; and (3) establish the traceability of regional and national enzyme methods to the IFCC reference methodologies (144). In the United States, the National Reference System for the Clinical Laboratory (NRSCL) Council of the NCCLS recognized the need for both reference methodology and materials when it instituted a formal consensus-accepted credentialing process for reference systems (135). In the case of enzymes, the credentialing process involves providing documentation to support a recognized and internationally accepted reference method, available reference materials, and documented transferability studies. To date, aspartate aminotransferase is, unfortunately, the only enzyme that has been credentialed by the NRSCL (145).

Use of the combination of reference methodology and materials to convert incompatible enzyme activity results for aspartate aminotransferase (146), alkaline phosphatase (147), and lactate dehydrogenase (148) to compatible values by use of a single scale, termed by the authors the International Clinical Enzyme Scale (ICES) has been suggested (149). Application of the ICES concept to the 1970 Scandinavian interlaboratory survey decreased the interlaboratory coefficient of variation from 38% to 16% for the enzymes tested. Similarly, in the 1971 New York State aspartate aminotransferase survey, the interlaboratory CV decreased from 41%-44% to 2%-5%, a major improvement. The Scandinavian Committee on Enzymes has expressed serious concerns about the philosophy of the ICES approach, and they again endorsed the widely accepted approach of ongoing development of reference methodologies and proper use of reference materials (150). Since this flurry of activity in 1984 and 1985, there has not been any further application or acceptance of the ICES concept.

7.3. Reference Materials for Enzymes

The requirements for, preparation of, and application of enzyme reference materials have been discussed extensively (134, 135, 151, 152, 153, 154, 155, 156, 157, 158). Cooperation among clinical enzymologists in Europe and the United States over the last several years has resulted in the availability of only a few enzyme reference materials. SRM 8430 from NIST is a preparation of human erythrocyte aspartate aminotransferase in a human albumin matrix (144); certified reference material (CRM) 319 from the Community Bureau of Reference (BCR) of the Commission of the European Communities is a preparation of porcine γ-glutamyltransferase in a bovine serum matrix (159). The working group of the BCR is in the process of establishing protocols and evaluating
reference materials for creatine kinase, alkaline phosphatase, and lactate dehydrogenase using LD-1 (143). Although not certified by any professional group involved with standardization, there are published reports detailing the preparation and characterization of potential reference materials that apparently meet the criteria of the NCCLS (157), and these include α-amylase (160), alkaline phosphatase (161) creatine kinase (162) and prostatic acid phosphatase (163). Reference materials 8025 and 8026 from the National Institute of Public Health and Environmental Hygiene in the Netherlands (164, 165) are secondary human-serum reference materials with target values assigned for aspartate and alanine aminotransferases, creatine kinase, and lactate dehydrogenase. NIST also has available a secondary lyophilized human serum reference material, SRM 909, with target values for aspartate and alanine aminotransferases, alkaline phosphatase, creatine kinase, lactate dehydrogenase, acid phosphatase, and γ-glutamyltransferase (166). Target values in these materials have been assigned using [IFCC reference methodology in round-robin type evaluations. SRM 909a-1 and 909a-2 from NIST is a two-level reference material that will be replacing SRM 909.

7.4. Standardization of Enzymes as Proteins

Immunological methods for enzymes, more specifically isoenzymes, such as lactate dehydrogenase-1 (167, 168), mitochondrial aspartate aminotransferase (169), prostatic acid phosphatase (170, 171, 172), and creatine kinase-MB (173, 174, 175), have been in use in the clinical laboratory for 10 years. However, the use of the immunological rather than catalytic properties of enzymes has not provided the opportunities for standardization that was anticipated a number of years ago (176, 177, 178). It is only within the last year that a working group on CK-MB mass assay was formed under the auspices of the Standards Committee of the American Association for Clinical Chemistry (AACC). The objective of this working group is to prepare a reference material to calibrate methods that are based on the principle of CK-MB mass measurement.

8. THE IDEAL AUTOMATED ENZYME ANALYZER

The “ideal” analyzer described here does not exist, although various manufacturers have some of the features on their devices, the qualities described here are those currently needed in contemporary automated enzyme assays in clinical laboratories. Our aim is to set some goals for future development in this area. Owing to patients’ needs and physicians’ demands, clinical laboratories must always be ready to accept and analyze specimens making them somewhat different from most other analytical services, moreover, redundancy and ruggedness are needed at all times. The current needs are broadly grouped into three areas: general human requirements, mechanical/computer needs, and analytical specifications.
8.1. General Requirements

The safety of laboratory personnel has become a major focus. Infectious agents in patient-derived materials pose a significant risk for laboratory workers, particularly HIV and hepatitis B. A clear goal is to reduce the exposure to infectious materials so that any contact is unlikely or even impossible. Of course, laboratory people are well indoctrinated on the use of universal precautions in handling specimens, but we must go beyond this to improve the failsafe nature of laboratory safety. Sampling out of a "closed" tube is a primary goal. Two areas of exposure are avoided: the aerosol that occasionally forms when a tube is opened, and possible contact with droplets in and around the top of the specimen container and where it is handled. Certainly, pouring the specimen into a cup or tube should be avoided; however, the technology of direct-collection-tube sampling is not generally available today, or the existing technology needs perfecting.

An analyzer should be environmentally friendly; the waste stream, both solid and liquid, should be minimal. Exposure to reagents should be absolutely minimized, and hazardous and/or carcinogenic reagents should be eliminated. Finally, all functions where human error are possible should be eliminated. A careful analysis of all the steps in the analytical chain must be made to identify nodes of possible human blunders.

8.2. Mechanical and Computer Needs

Specimen identification should be as infallible as possible. In an ideal system, the specimen collector (e.g., a phlebotomist) carries a portable computer and label generator. This person downloads the requisitions from the central laboratory system to their portable unit prior to specimen collection. At the collection point (e.g., at the bedside), the collector, using the portable unit, wands an identifying label (e.g., a bar code on a wrist band) from the source of the specimen (e.g., the patient); a bar-coded label is generated right there and is put on the specimen. Once in the laboratory, the label is "read" by the analyzer to give unambiguous identification. In the entire chain of analytical events, the specimen is always kept in the labeled container. Extra tubes, cups, and the like are never made. The central computer system downloads the tests to be performed to the instrument for a given patient, so there are no missed tests.

The instrument does automatic, reflex reassays for out-of-range specimens. Dilutions are prepared, a less-sensitive wavelength is used, or some other technique is implemented. In short, the final result is produced without operator intervention, calculations, or other operator manipulations. A "library" of specimens is prepared by the instrument to permit retrieval of specimens with special needs such as "add-on" tests, questions from users of the data, repeats, and so on.

The reagent systems are failsafe and are in bar-code-labeled containers that identify them, the lot, expiration date, and so on. It is impossible to use the
wrong reagent for a given test. The instrument monitors the inventory and quality of the reagents and alerts the operator to replenishment or maintenance needs.

Extensive safeguards exist for data maintenance including uninterruptable power supplies, redundant data storage, and retransmission capabilities to a host system where appropriate. The instrument also has extensive self-diagnostic features to monitor failures of all types including thermostatting, lamp failures, photometer misreadings of test materials, and so on.

8.3. Analytical Specifications

The specimen volume needed for analysis is minimal (e.g., 5µL), the “dead” volume in a specimen container is minimized, and the instrument has a “smart probe” as described above. Evaporation control is possible with lids of some kind, and a variety of different types of specimens, e.g., serum, plasma, urine, cerebrospinal fluid, and other body fluids can be accommodated on the same instrument. Obviously, the menu of enzyme tests must be broad enough to consolidate the testing on as few work stations as possible.

Finally, analytical accuracy, precision, and freedom from interferences must meet contemporary needs. Precision of modern instruments is largely excellent; bias from the true value is a common problem with enzyme assays, and interferences from lipemia, hemolysis, and icterus is still a problem with some instruments and methods.

9. APPENDIX 1: CHECKING ABSORBANCE ACCURACY WITH K₂Cr₂O₇/H₂SO₄ SOLUTIONS

Follow the procedure below:

A. Prepare 2 liters 0.005 mol/L H₂SO₄
   1. 0.05 mol/L H₂SO₄: 2.8 mL conc. H₂SO₄ (18 mol/L) Q.S. to 1 L with d. H₂O
   2. 0.005 mol/L H₂SO₄: Dilute 100 mL of 0.05 mol/L H₂SO₄ to 1 L with distilled H₂O. Stable indefinitely in glass. Label and date.
B. Dry about 15 g ACS or reagent-grade K₂Cr₂O₇ at 110°C for 2 hr. Cool in a desiccator.
C. Prepare a STOCK solution of 0.9350 g/L K₂Cr₂O₇ in 0.005 mol/L H₂SO₄. This solution has a theoretical absorbance (A) of 10.0 at 350 nm. Stable indefinitely in a well-stoppered, pyrex glass container.
D. Prepare dilutions of 2.5 mL-20 mL of the STOCK K₂Cr₂O₇ and enough 0.005 mol/L H₂SO₄ to make 100 mL of each dilution. The theoretical absorptivity is 10.7 L/g-cm.
10. APPENDIX 2: CALCULATING UNITS OF ENZYME ACTIVITY

An enzyme assay for LD (pyruvate to lactate direction) uses 0.010 mL serum in a total volume of 1.010 mL. The change in absorbance with time (slope) in the linear region of the reaction curve is 0.022/min. Calculate the enzyme activity in U/L. The molar absorptivity or ε of NADH is 6,220 L/mol-cm, b = 1 cm.

\[
\begin{align*}
1 \text{ U/L} & = 1 \text{ µmol/min-L substrate conversion under conditions of assay.} \\
\Delta c/\Delta t & = \text{µmol/min-L} \\
c & = A/\varepsilon b \\
\end{align*}
\]

Therefore:

\[
\frac{\Delta A}{\Delta t} \cdot \frac{1}{\varepsilon b} \cdot \frac{TV}{SV} = \text{U/L}
\]

\[
\frac{0.022}{\text{min}} \cdot \frac{1 \text{ mol-cm}}{6220 \text{ L}} \cdot \frac{1}{1 \text{ cm}} \cdot \frac{1.01}{0.01} \cdot \frac{10^6 \text{ µmol}}{\text{mol}} = 357 \text{ U/L}
\]

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