**Experimental Engineering Section Semiannual
Progress Report for the Period March 1 to
August 31, 1976**

**Volume 2: Biotechnology and
Environmental Programs**

MASTER

OAK RIDGE NATIONAL LABORATORY
OPERATED BY UNION CARBIDE CORPORATION · FOR THE DEPARTMENT OF ENERGY

DISTRIBUTION OF THIS DOCUMENT IS UNLIMITED

~~DISTRIBUTION OF THIS DOCUMENT IS LIMITED
TO ERDA OFFICES AND ERDA CONTRACTORS~~

DISCLAIMER

This report was prepared as an account of work sponsored by an agency of the United States Government. Neither the United States Government nor any agency Thereof, nor any of their employees, makes any warranty, express or implied, or assumes any legal liability or responsibility for the accuracy, completeness, or usefulness of any information, apparatus, product, or process disclosed, or represents that its use would not infringe privately owned rights. Reference herein to any specific commercial product, process, or service by trade name, trademark, manufacturer, or otherwise does not necessarily constitute or imply its endorsement, recommendation, or favoring by the United States Government or any agency thereof. The views and opinions of authors expressed herein do not necessarily state or reflect those of the United States Government or any agency thereof.

DISCLAIMER

Portions of this document may be illegible in electronic image products. Images are produced from the best available original document.

Printed in the United States of America. Available from
National Technical Information Service
U.S. Department of Commerce
5285 Port Royal Road, Springfield, Virginia 22161
Price: Printed Copy \$10.75; Microfiche \$3.00

This report was prepared as an account of work sponsored by an agency of the United States Government. Neither the United States Government nor any agency thereof, nor any of their employees, contractors, subcontractors, or their employees, makes any warranty, express or implied, nor assumes any legal liability or responsibility for any third party's use or the results of such use of any information, apparatus, product or process disclosed in this report, nor represents that its use by such third party would not infringe privately owned rights.

Contract No. W-7405-eng-26

CHEMICAL TECHNOLOGY DIVISION

T - EXPERIMENTAL ENGINEERING SECTION SEMIANNUAL PROGRESS REPORT
FOR THE PERIOD MARCH 1 TO AUGUST 31, 1976

VOLUME 2: BIOTECHNOLOGY AND ENVIRONMENTAL PROGRAMS

Compiled by:

W. W. Pitt, Jr.
J. E. Mrochek

2 comp.

NOTICE

This report was prepared as an account of work sponsored by the United States Government. Neither the United States nor the United States Department of Energy, nor any of their employees, nor any of their contractors, subcontractors, or their employees, makes any warranty, express or implied, or assumes any legal liability or responsibility for the accuracy, completeness or usefulness of any information, apparatus, product or process disclosed, or represents that its use would not infringe privately owned rights.

Date Published: March 1978

NOTICE This document contains information of a preliminary nature. It is subject to revision or correction and therefore does not represent a final report.

OAK RIDGE NATIONAL LABORATORY
Oak Ridge, Tennessee 37830
operated by
UNION CARBIDE CORPORATION
for the
DEPARTMENT OF ENERGY

DISTRIBUTION OF THIS DOCUMENT IS UNLIMITED

~~DISTRIBUTION OF THIS DOCUMENT IS LIMITED~~
~~To ERDA Offices and ERDA Contractors~~

THIS PAGE
WAS INTENTIONALLY
LEFT BLANK

CONTENTS

	<u>Page</u>
SUMMARY	vii
1. ENVIRONMENTAL RESEARCH	1
1.1 Automated Analysis of Dissolved Organics in Polluted Waters	2
1.1.1 Aqueous process stream from a coal conversion process	2
1.1.2 Analysis of organics in drinking water	5
1.1.3 Analysis of organics in goitrogenic well water	5
1.1.4 Preliminary evaluation of concentration methods for organic constituents in water samples.	7
1.2 Continuous Monitoring of Pollutants	9
1.2.1 Continuous chemical oxygen demand monitor.	10
1.2.2 Fluorometric monitor	10
1.2.3 Aquatic environmental monitor.	14
1.3 Environmental Effects of Antifoulants	18
1.4 Assessment of Environmental Control Technology for Coal Hydrocarbonization	20
1.5 References for Section 1.	30
2. CENTRIFUGAL ANALYZER DEVELOPMENT	33
2.1 Multipurpose Optical System	34
2.1.1 Multipurpose optical system configuration.	34
2.2 Development of Portable Centrifugal Fast Analyzer	42
2.2.1 Description of system.	42
2.2.2 Microprocessor control of the portable Centrifugal Fast Analyzer.	45
2.2.3 Temperature control.	61
2.2.4 The portable Centrifugal Analyzer - a precision spectrophotometer.	81
2.2.5 Fast transfer and mixing	81
2.2.6 Concept for documentation of mechanical components	84
2.2.7 Inertial response in rotating systems.	88
2.3 Applications.	102
2.3.1 Development of Centrifugal Fast Analyzer rotors preloaded with reagent	102

	<u>Page</u>
2.3.2 Creatine phosphokinase isoenzyme measurement . . .	.112
2.3.3 Biochemical markers of cancer - measurement of gamma-glutamyl transpeptidase, leucine aminopeptidase, and 5'-nucleotidase.	.132
2.3.4 Monitoring kynurenine formamidase activity in mouse liver homogenates.	.147
2.3.5 Adaptation of Coombs testing to the Centrifugal Fast Analyzer.	.149
2.3.6 Environmental analysis	.159
2.4 References for Section 2.	.169
3. ADVANCED ANALYTICAL SYSTEMS.	.175
3.1 High-Resolution Liquid Chromatographic Systems.	.175
3.1.1 Chromatographic analysis for protein-bound carbohydrates in normal femal subjects and breast-cancer patients	.175
3.1.2 High-resolution chromatographic separation of isoenzymes.	.178
3.2 Blood Sample Preparation System	.181
3.2.1 Background	.181
3.2.2 Results for a third-generation prototype system. . .	.182
3.2.3 Design and fabrication of new prototype system . .	.184
3.2.4 Sample collection.	.186
3.3 Automated Elution Electrophoresis	.192
3.3.1 Transport phenomena: current and joule heating. .	.192
3.3.2 Experimental objectives.	.195
3.3.3 Equipment and materials.	.196
3.3.4 Experimental procedure	.199
3.3.5 Results and discussion	.200
3.3.6 Recommendations.	.202
3.4 References for Section 3.	.203
4. BIOENGINEERING RESEARCH.	.205
4.1 Enzyme Catalysis.	.206
4.1.1 Purification and separation of ferredoxin and hydrogenase.	.207
4.1.2 Enzyme stabilization and immobilization.	.213
4.1.3 Rate of oxidation of ferredoxin.	.214

	<u>Page</u>
4.2 Tritium Isolation by Bacteria	217
4.3 References for Section 4.	219
5. BIOENGINEERING DEVELOPMENT	221
5.1 ANFLOW Bioreactor	223
5.1.1 Pilot plant development.	226
5.1.2 Supporting research.	230
5.2 Biotreatment of Coal Conversion Aqueous Effluents	233
5.3 ORNL Support of the Nitrate Waste Recycle Facility at Y-12.	238
5.3.1 Task I - backup storage of seed bacteria	238
5.3.2 Task II - bacterial growth during reactor start-up	240
5.3.3 Task III - determination of uranium and plutonium balance in bioreactor sludge	243
5.4 Tapered Fluidized-Bed Bioreactor Studies.	245
5.4.1 Ethanol production	245
5.4.2 Variable Angle Test Unit	250
5.5 References for Section 5.	253

**THIS PAGE
WAS INTENTIONALLY
LEFT BLANK**

SUMMARY

This volume contains the progress report of the biotechnology and environmental programs in the Experimental Engineering Section of the Chemical Technology Division. Research efforts in these programs during this report period have been in five areas:

1. Environmental research;
2. Centrifugal analyzer development;
3. Advanced analytical systems development;
4. Bioengineering research; and
5. Bioengineering development.

Summaries of these programmatic areas are contained in Volume I.*

*C. D. Scott et al., Experimental Engineering Section Semiannual Progress Report for the Period March 1 to August 31, 1976. Volume I: Summary Report, ORNL/TM-5865/V1 (September 1977).

1. ENVIRONMENTAL RESEARCH

Environmental research efforts during this report period have been in four areas:

1. Automated analysis of dissolved organics in polluted waters;
2. Continuous monitoring of pollutants;
3. Environmental effects of antifoulants; and
4. Assessment of environmental control technology for coal conversion processes.

During this report period, an aqueous process stream from a coal conversion process, a chlorinated drinking water, and a drinking water from an area having a high incidence of endemic goiter have been examined by high-resolution liquid chromatography (HRLC).

Two continuous environmental monitors are currently under development: (1) a continuous chemical oxygen demand (CCOD) monitor, which is particularly sensitive to phenolic compounds; and (2) a continuous flow fluorometer with a wide dynamic range for monitoring dissolved polycyclic aromatic hydrocarbons (PAHs). Both instruments are being incorporated into a prototype system for monitoring aquatic pollutants. During this report period, the effects of sample-to-reagent Ce(IV) concentration in the CCOD monitor were investigated using phenol as a stand-in pollutant.

The bioaccumulation and incorporation of chloro-organics are a matter of environmental concern; therefore, as part of the program on the environmental effects of antifoulants, several test species obtained below chlorinated water outfalls will be examined for incorporation of chloro-organic compounds. In an initial experiment conducted during this report period, 60 g of *Chironomus riparius*, a bloodworm, was collected below the

chlorinated outfall of the Oak Ridge West Waste Treatment Plant. The DNA from these animals were isolated and hydrolyzed, and nucleosides were separated; however, the results were inconclusive because an inactive RNAase enzyme was inadvertently used in the DNA isolation. The observation suggests the tentative conclusion that the nucleoside, 5-chlorodeoxyuridine, was not present in the DNA hydrolysate. During this report period, the wastewater problem for a commercial hydrocarbonization plant based on the Coalcon design was defined, and an interim report was drafted.

1.1 Automated Analysis of Dissolved Organics in Polluted Waters

W. W. Pitt, Jr., R. L. Jolley, G. Jones, Jr., J. E. Thompson

The major objective of this program is to develop methods for analyzing soluble organic compounds in waters of environmental concern. Data generated from this program should assist in the gathering of quantitative and qualitative information concerning water pollutants which is necessary to understand possible health and environmental effects. Efforts during this research period have been concentrated on examining an aqueous process stream from a bench-scale-unit coal conversion process, evaluating a preconcentration technique to facilitate the HRLC analysis of microgram-per-liter concentrations of organics present in complex mixtures in waters, examining a drinking water from an area having a high incidence of endemic goiter, and conducting a preliminary comparison of preconcentration techniques for wastewater.

1.1.1 Aqueous process stream from a coal conversion process

The limited supply of natural gas and petroleum has focused national attention on the conversion of coal and oil shale to liquid and gaseous

hydrocarbons as an alternate source for fuel and petrochemicals. Each coal conversion technology, at some stage of the process, produces water during the heating and decomposition of the coal. These aqueous streams, analogous to the ammoniacal liquor waste from the coking industry, may be recycled but must ultimately be considered for treatment in a pollution abatement process before their release to the environment. The chemical composition of these aqueous process streams is of interest with respect to understanding possible health effects caused by inadvertent releases within the plant environs, developing waste treatment processes, and determining the potential environmental impact associated with the possible discharge of such aqueous streams to the environment.

In the Advanced Technology Section of the Chemical Technology Division, a bench-scale study of the coal hydrocarbonization process is being conducted.¹ As part of this study, the organic constituents in the aqueous stream from the product scrubber are being characterized using HRLC for separation of the water soluble organics and gas chromatography--mass spectrometry (GC/MS) for identification of the organics.^{2,3} In the first preparative-scale HRLC separation, more than 100 uv-absorbing constituents and 50 cerate-oxidizable constituents were detected and separated. Twenty of the chromatographic peaks were apparently common. Several of the chromatographic peaks were "off-scale," indicating rather large concentrations of organics in the aqueous sample. Resorcinol, orcinol, hydroquinone, and a dihydroxyxylene isomer were identified by GC and MS. In addition, phosphate, glycerine, O-methylinositol, palmitic acid, oleic acid, linoleic acid, and stearic acid were identified. Mass spectra were obtained on six additional compounds (Table 1.1).

Table 1.1. Gas chromatographic and mass spectral characteristics of unknown organic constituents in an aqueous process stream from a coal hydrocarbonization bench-scale unit

Estimated molecular weight	Anion exchange elution fraction	Gas chromatographic retention position ^a (OV-1)	Mass spectral properties ^b				
			M	Base(1)	(2)	(3)	(4)
73	162-170	11.5	217	174	202	128	86
102	2-5	10	89	100	174	86	143
118	2-5	11	262	174	262	100	224
126	2-5	-	270	215	173	265	149
136	95-111	11	280	215	192	170	188
137	2-5	-	209	194	209	120	141

^a Methylene unit (MU) retention position of the trimethylsilyl (TMS) derivative.

^b Molecular ion and principal peaks are listed. All the peaks with m/e less than 73 and the silicon-containing peaks which are common to most mass spectra of TMS derivatives (i.e., 147, 79, 77, 75, and 73) are not listed.

1.1.2 Analysis of organics in drinking water

Relatively little data exist concerning the nonvolatile organic constituents in drinking water. Because of the determination that toxic, volatile chlorinated organics are formed in chlorinated drinking water (e.g., chloroform), some investigators have expressed concern that non-volatile organics, either chlorinated or unchlorinated, may present similar problems. Thus, several preliminary studies have been conducted on drinking water samples using ion exchange cartridges for concentration of the organics and HRLC for separation and detection.

In one HRLC separation of the organic constituents in a 5-liter aliquot of chlorinated drinking water from a metropolitan water treatment plant, eight uv-absorbing constituents were separated. Four of these chromatographic peaks were large, and one of these represented a rather massive amount of material. In another HRLC separation (a preparative-scale run) of the organic constituents in a 40-liter aliquot of chlorinated drinking water from a small municipal water treatment plant, only one uv-absorbing constituent was detected, but several cerate-oxidizable constituents were separated. Using a multicomponent identification method,^{2,3} phosphate, glycerine, O-methylinositol, benzoic acid, palmitic acid, oleic acid, linoleic acid, and stearic acid were identified among the separated constituents. In addition, ten unknown constituents were characterized with respect to gas chromatographic and mass spectral properties (Table 1.2).

1.1.3 Analysis of organics in goitrogenic well water

Waterborne goitrogens are being investigated as causative factors of endemic goiter in western Colombia. In collaboration with Dr. Eduardo Gaitan, Professor of Medicine, Universidad del Valle, Cali, Colombia, we

Table 1.2. Gas chromatographic and mass spectral characteristics of unknown organic constituents in a municipal drinking water sample

Estimated molecular weight	Anion exchange elution fraction	Gas chromatographic retention position ^a		Mass spectral properties ^b				
		OV-1	OV-17	M	Base(1)	(2)	(3)	(4)
113	181-134	12.3	12.3	257	188	144	170	120
113	181-134	12.8	12.6	257	120	170	188	192
149	196-201	12	-	293	278	264	130	146
168	109-114	11.8	12.9	312	225	146	130	105
170	196-201	16.8	18.6	314	295	140	299	163
202	191-195	19.3	18.5	346	253	295	331	299
210	196-201	16.2	18.5	282	149	177	267	223
245	109-114	13.8	15.7	389	227	131	133	115
276	185-190	16.0	18.6	420	405	327	156	343
-	196-201	17.0	19.0	-	217	299	317	405

^a Methylene unit (MU) retention position of the trimethylsilyl (TMS) derivative.

^b Molecular ion and principal peaks are listed. All the peaks with m/e less than 73 and the silicon-containing peaks which are common to most mass spectra of TMS derivatives (i.e., 147, 79, 77, 75, and 73) are not listed.

have examined several methanol and ether extracts of a goitrogenic well water from Candelaria, Columbia. A HRLC analysis of the samples revealed rather unresolved large peaks which were probably humic materials. Gas chromatographic analysis of the methylene chloride extract of the concentrate with the highest antithyroid activity gave a complex chromatogram with several large peaks. Mass spectral data for these peaks are given in Table 1.3. Although these spectra were compared with several library files of mass spectral data, none of the organics were identified.

1.1.4 Preliminary evaluation of concentration methods for organic constituents in water samples

The evaluation of the concentration of organics from water samples using ion exchange sorption methods as reported previously⁴ is being continued. This process involves: (1) adjustment of the pH of the water sample to 8.5, (2) passing the sample through an anion exchange cartridge (0.62-cm-ID by 4-in. stainless steel column filled with AG 1-X8 resin, $\geq 40\mu$), (3) adjustment of the pH of the eluate to 4.0, and (4) passing the sample through a cation exchange cartridge (0.62-cm-ID by 4-in. stainless steel column filled with AG 50-WX12 resin, $\geq 20\mu$). The resin cartridges are then placed on either an anion or cation exchange high-resolution system, and the organic constituents are chromatographically separated as in a routine chromatographic run.

A 50-liter water sample was collected from the secondary effluent of the Oak Ridge East Waste Treatment plant prior to disinfection of the effluent with chlorination. Half the sample was concentrated using vacuum distillation followed by lyophilization. The other half, 25 liters, was passed through an anion exchange cartridge after pH adjustment to 9.0. Because of the precipitation of a flocculent material, probably iron

Table 1.3. Gas chromatographic and mass spectral characteristics of unknown organic constituents in a goitrogenic well water sample^a

Estimated molecular weight	Gas chromatographic retention position ^b (OV-1)	Mass spectral properties ^c				
		M	Base(1)	(2)	(3)	(4)
89 ^d	13.43	233	117	118	119	219
89 ^d	13.61	233	117	118	119	219
112	11.6	256	99	187	191	241
115	12.8	331	141	283	211	145
116	15.22	332	144	188	117	202
169	~16.3	385	145	192	117	164
194	16.0	410	166	124	96	117
202	~18	346	99	155	169	195
235	~19	379	155	207	111	148

^aA methanol and ether extract from goitrogenic well A of Candelaria, Colombia, by courtesy of Dr. Eduardo Gaitan, Professor of Medicine, Universidad del Valle, Cali, Colombia.

^bTMS derivatives.

^cMolecular ion and principal peaks are listed. All the peaks with m/e less than 73 and the silicon-containing peaks which are common to most mass spectra of TMS derivatives (i.e., 147, 79, 77, 75, and 73) are not listed.

^dIsomers.

hydroxide, pH 9 was the maximum pH that could be used. Plugging of the cartridge and a gradual decrease in flow rate through the cartridge were still experienced apparently due to the finely divided organic material which was not removed by prefiltration through filter paper. Concentration with a cation exchanger at pH 4 was achieved without any difficulty. A HRLC analysis of the two comparative samples, the vacuum distillation concentrate and the anion exchange cartridge concentrate, was aborted because of overloading the analytical columns. Subsequent HRLC analysis of the cation exchange cartridge concentrate indicated that the elution of calcium and the resulting precipitation of calcium sulfate in the cerate oxidimetry system could be a major problem with this method. Although these methods will be further compared, the method of vacuum distillation will probably be selected for an impending investigation of the nonvolatile organics in several wastewater effluents.

1.2 Continuous Monitoring of Pollutants

The development of continuous monitoring systems for sensitive onsite monitoring of dissolved aquatic pollutants constitutes a very practical, necessary approach to one facet of our growing concern for the perturbation in our environment caused by various anthropogenic energy sources. The objective of this program is the development of such monitoring systems through the design, fabrication, and evaluation of prototype instruments. Two instruments are currently under development: (1) a CCOD monitor, which is particularly sensitive to phenolic compounds; and (2) a continuous flow fluorometer with a wide dynamic range for monitoring dissolved PAHs. Both instruments are being incorporated into a single system for monitoring waste streams from coal processing plants.

1.2.1 Continuous chemical oxygen demand monitor

The CCOD monitor is based on cerate oxidimetry; that is, the measurement of the fluorescent Ce(III) resulting from the reduction of Ce(IV) by any oxidizable compound in the stream being monitored.⁵ During this report period, the effects of sample-to-reagent flow ratio and reagent Ce(IV) concentration were investigated using phenol as a stand-in pollutant. It can be seen from Fig. 1.1 that, although the sensitivity of the CCOD monitor increases with decreasing Ce(IV) concentration, the linear range decreases. A reagent concentration of 5 mM Ce(IV) was selected as the best compromise, providing the maximum sensitivity with a linear response up to 10 µg/ml of phenol. Using a reagent of this concentration and a sample containing 1 µg/ml of phenol, the effect of the sample-to-reagent ratio on instrument response was determined (Fig. 1.2).

1.2.2 Fluorometric monitor

Since many of the organic contaminants (particularly PAHs) found in wastewaters and the aquatic environment are strongly fluorescent, a continuous flow fluorometer for monitoring aquatic pollutants is being developed. Two different instrumental concepts are being investigated: (1) a bifurcated quartz fiber optic (BFO) fluorometer;⁴ and (2) a small, conventional, right-angle fluorometer with a quartz tube flow cell. During this report period, we have concentrated on using the fluorometers as monitors for dissolved PAHs, since they are both highly fluorescent and potentially very hazardous even at very low concentrations.

Initially, a qualitative study of the relative solubilities and fluorescences of several PAHs was made, and the results are shown in Table 1.4. The data in Table 1.4 were obtained in the following manner.

Figure 1.1. Effect of increasing Ce(IV) concentration on the sensitivity and linear range of the continuous chemical oxygen demand monitor.

Figure 1.2. Effect of the sample-to-reagent ratio on the response of the continuous chemical oxygen demand monitor.

Table 1.4. Qualitative solubilities and relative fluorescence of polycyclic aromatic hydrocarbons

Compound	Initial methanol solution ^a (mg/liter)	Solubility in water using methanol as carrier (mg/liter)	Relative fluorescence ^b λ_{Ex} , 254 nm λ_{Em} , >320 nm
Pyrene	500	5	1.0
Phenanthrene	500	5	0.6
3,4-dimethylphenol	200	100	0.05 ^c
Naphthalene	500	250	0.22 ^c
2,3-dimethylnaphthalene	1000	10	0.70
2-methylnaphthalene	1000	500	0.50 ^c
Adamantane	200	0	
2,3-benzofluorene	100	50	2.8
Triphenylene	500	15	0.008
Retene	320	10	0.05
2,3-benzindene	900	25	0.56
Fluorene	900	5	2.2
Fluoranthene	250	1	0
Biphenyl	500	250	0.8 ^c
Chrysene	25	0	
Dibenzofuran	750	0	
Rubrene	25	10	0.1 ^c
Perylene	10	5	1.8 ^c
1,2-benzofluorene	100	50	4.2 ^c
Anthanthrene	100	50	0 ^c
1,2-benzanthracene	500	100	0.75
1,2,3,4-dibenzanthracene	50	5	2.0
3,4-benzopyrene	75	15	1.1
2,3,6,7-dibenzanthracene	20	10	0.2 ^c

^aValues <100 mg/liter represent approximate solubility of compound in methanol.

^bPyrene chosen as standard (1 mg/liter = 1 mV detection response).

^cBackground fluorescence of methanol subtracted.

Each compound was initially dissolved in methanol, and this solution was diluted (beginning with a twofold dilution) with distilled water until no precipitation occurred. The fluorescences of the resulting solutions were measured with the BFO fluorometer using 254-nm excitation and a 0-53 Corning filter as the secondary filter. The most highly fluorescing compound, 2,3-benzofluorene, was used to test the response of the fluorometer with the quartz tube flow cell. This fluorometer had a measurable response to 0.1 $\mu\text{g/liter}$ and was linear over the range between 2 and 2000 $\mu\text{g/liter}$ of 2,3-benzofluorene (Fig. 1.3). For comparison, the response of the bifurcated optic fluorometer to phenanthrene (fluorescence is approximately one-fifth that of 2,3-benzofluorene) is shown in Fig. 1.4. Although the sensitivities of the two instruments are comparable, the response of the BFO is highly nonlinear. This results from the nonrandomly mixed nature of the fiber optics. It is expected that the nonlinearity will be reduced significantly through the use of randomly mixed fiber optics in the second version of the BFO fluorometer.

1.2.3 Aquatic environmental monitor

Both the CCOD monitor and the flow fluorometer are being incorporated in a prototype aquatic environmental monitor (Fig. 1.5) which is under construction. Although the instrument is not complete, it has been used to determine the level of pollution of secondary sewage effluent from the Oak Ridge East Waste Treatment Plant before and after chlorination. The effluent was found to contain 9 $\mu\text{g/ml}$ of chemical oxygen demand (COD) both before and after chlorination, but the fluorescence was reduced $\sim 20\%$ by chlorination (Table 1.5).

Figure 1.3. Response of the bifurcated optic fluorometer to phenanthrene.

Figure 1.4. Response of the commercial, right-angle fluorometer to 2,3-benzofluorene.

CCOD SYSTEM

REAGENT - PERCHLORATO-CERIC
ACID

EXCITATION - 254 nm INTERFERENCE FILTER

EMISSION - CORNING 7-60 FILTER

Figure 1.5. Aquatic environmental monitor.

Table 1.5. Organic contamination in the Oak Ridge East Waste Treatment Plant secondary effluent

	COD ($\mu\text{g/ml}$)	Fluorescence (mV) ^a
Before chlorination	9	38
After chlorination	9	30

^a1 mV corresponds to the fluorescence of 1×10^{-5} M Ce(III).

1.3 Environmental Effects of Antifoulants

W. W. Pitt, Jr., R. L. Jolley, and J. E. Thompson

Chlorine is the principal biocide for antifoulant treatment of cooling systems for electric power-generating plants. We have previously shown that chloro-organic compounds are formed during the chlorination of cooling waters and process effluents.⁶⁻⁸

Two 40-liter aliquots of Kingston Steam Plant cooling waters, one prior to chlorination and the second after chlorination to 0.2 ppm chlorine residual, were passed through anion and cation exchange cartridges to evaluate the efficacy of that method for concentrating the organic constituents for HRLC analysis.⁴ Chromatography of the two anion exchange cartridges on the preparative-scale UV Analyzer indicated that relatively few uv-absorbing constituents were extracted from the waters by the resin cartridges. In each case, several cerate-oxidizable constituents were separated. The fractions collected from the HRLC analysis of the chlorinated cooling water sample are being processed through the multicomponent identification scheme.^{2,3}

Bioaccumulation and incorporation of chloro-organics in the biota is a matter of environmental concern. Therefore, several test species obtained

below the chlorinated cooling water outfalls will be examined for incorporation of chloro-organic compounds. The pyrimidine base, 5-chlorouracil, has been tentatively identified in chlorinated wastewater treatment plant effluents and in chlorinated cooling waters.⁶⁻⁸ R. B. Cumming and co-workers have shown that 5-chlorouracil is incorporated into the DNA of the bacteria *Escherichia coli* and mice apparently as the thymine analog.⁹⁻¹¹ Therefore, two test species, *Chironomus* sp. and *Corbicula* sp., have been selected for preliminary examination. *Chironomus* sp. in the larvae stage are known as bloodworms and live in the bottom sediments of streams and ponds. *Corbicula* sp. are filter feeding clams that often physically restrict the flows into cooling water intakes and intakes for water treatment plants.

Sixty grams of *Chironomus riparius*, the bloodworm below the chlorinated sewage outfall of the Oak Ridge West Waste Treatment Plant, was collected by Blaylock and co-workers of the Environmental Sciences Division. The nonbiting midge larvae were purged for 24 hr in freshwater to eliminate the gut contents and then frozen at -60°C. The DNA was separated from the animals using the method developed by J. Marmur.¹² The essential steps in this method are the following: (1) grind tissue to powder in liquid nitrogen, (2) lyse tissue cells in aqueous solution of sodium dodecylsulfate, (3) denature protein with chloroform--octanol 10/1 by volume), (4) centrifuge to remove precipitated protein, (5) remove supernate and wash twice with chloroform--octanol solution, (6) precipitate DNA with cold ethanol, (7) dissolve DNA in sodium chloride--sodium citrate solution, (8) incubate with RNAase, trypsin, and pronase enzymes, sequentially, (9) extract DNA with saturated phenol solution (0.03 M at pH 7.4),

(10) remove phenol by ether extraction, and (11) precipitate purified DNA with cold ethanol. The isolated DNA was hydrolyzed with DNAase-I, snake-venom phosphodiesterase, and alkaline phosphatase. Chromatography on Aminex A-6 cation exchange resin was used to separate the DNA hydrolysate nucleosides. The first effort was inconclusive because of gross RNA nucleoside contamination, thus indicating that the RNAase enzyme used was not active. However, an observation suggests the tentative conclusion that the nucleoside 5-chlorodeoxyuridine was not present in the DNA hydrolysate. We wish to acknowledge the assistance of M. Walton for the DNA extraction and hydrolysis and B. Pal for the chromatographic analysis. Further studies will be made of the possible incorporation of chloro-organics in bloodworms and also *Corbicula* sp. Specifically, they will be examined not only for incorporation of DNA bases but also lipophilic chloro-organics using methodology developed by P. M. Williams and K. J. Robertson.¹³

1.4 Assessment of Environmental Control Technology for Coal Hydrocarbonization

J. A. Klein and R. E. Barker

In order to commercialize the production of clean (i.e., low-sulfur) boiler fuel from coal, DOE and Coalcon have contracted to develop hydrocarbonization to the demonstration scale. This report represents an attempt to define the wastewater problem for a commercial hydrocarbonization plant using the Coalcon design.¹⁴ The disposal of wastewater from coal conversion plants is not a trivial problem. Approximately 80 large (12,000 to 20,000-tons/day) coal conversion plants will be required to meet the goal of replacing 20% of the present U.S. oil consumption with clean fuels from coal.¹⁵ A coal conversion industry of this size would produce a very large amount of wastewater (400×10^6 to 800×10^6 tons/year).

Furthermore, hydrocarbonization is one of the best-developed coal conversion technologies, with the Coalcon demonstration now in the design and construction phase. Hydrocarbonization is a likely process for commercialization for three main reasons:

1. Only premium, low-sulfur gaseous and liquid products are produced;
2. Hydrocarbonization is a noncatalytic process; and
3. There is no difficult solid-liquid separation.

For these reasons, an environmental evaluation of hydrocarbonization is a timely project.

At the time of this writing, there has been no environmental evaluation of the hydrocarbonization process published. However, evaluations of other processes have been published. Exxon has published evaluations of several gasification and liquefaction processes¹⁶⁻²⁶ in conjunction with the U.S. Environmental Protection Agency. Booz-Allen Applied Research has also published, under contract with the EPA, an evaluation of several clean fuel processes.²⁷ Several other workers have published coal conversion environmental evaluations.²⁸⁻³¹

Coal conversion aqueous wastes are often compared with their counterpart in the coke production industry. For this reason, the phenolic and light oil components of the waste are well characterized and have, in general, been well considered. Hydrocarbonization will produce greater quantities of these pollutants and will have more serious ammonia, hydrogen sulfide, and trace element problems.

Some 71 process streams involved in the Coalcon hydrocarbonization process have been identified, and their potential for wastewater management has been evaluated. These streams are shown in Fig. 1.6 and described in Table 1.6.

Figure 1.6. Schematic hydrocarbonization plant.

Table 1.6. Identification of Coalcon process streams

Stream No.	Stream Description
1	Coal from mine, 628 tons/hr
2	Rainwater to coal storage
3 ^a	Rainwater runoff from coal storage, 530 lb/hr
4	Coal feed to crushing, 628 tons/hr
5	Coal to boiler, 35.1 tons/hr
6	Coal to hydrocarbonization, 593 tons/hr
7	Dry flue gas to coal drying, 1,080,428 lb/hr
8	Wet flue gas to sulfur recovery, 1,092,112 lb/hr
9	Steam from char cooling, 5000 lb/hr
10	Contaminated condensate, 125,000 lb/hr (steam made from contacting water with hot char)
11	Steam, 60,642 lb/hr
12	Pressurized coal, 1,089,167 lb/hr
13	Heavy fuel oil to storage, 211,779 lb/hr
14	Water (to be converted to steam by char contact)
15	Light fuel oil, 78,936 lb/hr
16	Char and transport steam: 5000 lb/hr steam, 513,144 lb/hr char
17	Hydrogen gas, 208,106 lb/hr
18	Light gases from reactor, 397,069 lb/hr
19 ^a	Reactor aqueous product to ammonia stripping, 105,056 lb/hr
20	Steam from hydrocarbonization to pressurization, 85,274 lb/hr
21	Agglomerates, ~160 lb/hr char
22	Ammonia-rich vapor stream to ammonia recovery
23 ^a	Condensate recycle, 12,331 lb/hr
24	Ammonia product, 4,067 lb/hr
25 ^a	Wastewater from ammonia stripping to char slurrying, 175,000 lb/hr
26	Gas from ammonia stripping to acid-gas removal, 472,000 lb/hr (ethane, methane, H ₂ S, etc.)
27 ^a	Wastewater recycle, 52,000 lb/hr

Table 1.6. (continued)

Stream No.	Stream Description
28	Steam, 18,000 lb/hr
29	H ₂ S-rich stream to sulfur recovery, 13,300 lb/hr
30	Steam, 20,000 lb/hr
31	Water, 32,000 lb/hr
32	Cleaned gas to hydrogen purification, 380,000 lb/hr
33	LPG product to storage, 34,600 lb/hr
34	Synthesis gas to methanation, 168,917 lb/hr
35	Hydrogen recycle to reactor, 32,895 lb/hr
36	SNG product to storage, 206,282 lb/hr
37 ^a	Wastewater, 38,000 lb/hr
38	Makeup hydrogen from Texaco unit, 162,865 lb/hr
39	Char-water slurry to Texaco unit: 354,663 lb/hr char, 250,292 lb/hr water
40	Makeup water to char slurring, ~75,000 lb/hr
41	Sluicewater return from ash pond
42 ^a	Ash slurry to pond from Texaco unit: 86,581 lb/hr, 10% H ₂ O
43 ^a	Gasifier water jacket blowdown, 1,715 lb/hr
44	Steam, 70,000 lb/hr
45	Makeup water, 68,430 lb/hr
46	Quench evaporation
47	Char to Texaco unit, 354,663 lb/hr
48	Char to boiler, 158,481 lb/hr
49	Boiler flue gas to sulfur recovery, 1,574,284 lb/hr
50	Sulfur recovery flue gas feed, 2,666,396 lb/hr
51	Desulfurized flue gas to stack, 3,527,940 lb/hr
52	Ash sluicewater return, 308,331 lb/hr
53 ^a	Ash slurry to ash pit, 34,259 lb/hr ash, 308,331 lb/hr water
54	Sluicewater makeup
55	Boiler feed water (BFW), 1,623,800 lb/hr
56	Steam, 1,593,800 lb/hr

Table 1.6. (continued)

Stream No.	Stream Description
57 ^a	Boiler blowdown, ~30,000 lb/hr
58 ^a	Contaminated rainwater runoff of plant grounds
59 ^a	Cooling tower blowdown, 358,000 lb/hr
60	Cooling tower losses
61	Cooling tower makeup, 4,081,000 lb/hr
62	River water, 5,658,500 lb/hr
63	BFW, 3,410,000 lb/hr
64	Potable water, 150,000 lb/hr
65	Wastewater to Texaco unit, 50,000 lb/hr
66 ^a	Clarifier blowdown, 70,000 lb/hr
67 ^a	Blowdown from demineralizer, 265,000 lb/hr
68	Water to ammonia recovery, 89,300 lb/hr
69	Turbine condensate
70	BFW return
71	Steam

^aDenotes wastewater stream.

In conclusion, 15 wastewater streams were identified, and coal conversion literature has been surveyed for the purpose of predicting the characteristics of these streams. Table 1.7 is a summary of the characteristics of the Coalcon wastewater streams.

Theoretically, many of the wastewater streams will be completely recycled internally and thus may not be of concern. Others are fairly well defined, and adequate chemical technology exists to bring these streams into compliance with any reasonable regulations.

However, a number of streams are not well characterized either in quantity of flow or in pollutant content. This is particularly true of the organic content (PAHs, phenols, etc.) of the cooling tower streams and the trace element content of the ash slurry to the ash pit. These streams will also be the ones that will most likely present some problems in the application of adequate cleanup technology. Therefore, the following recommendations are given.

1. Additional research should be conducted on the characterization of critical wastewater streams including: (1) coal storage and plant ground runoff, (2) water streams from the hydrocarbonization reactor, ammonia stripping, and condensate from the hydrocarbon separation unit, and (3) ash and slag quenching and sluicing water and leachate from stored ash and slag.
2. Additional reliability studies should be conducted to quantify leak rates through the many heat exchangers that will be needed in a coal conversion plant.
3. Future research studies should be conducted to determine safe effluent limits for PAHs and a variety of trace elements. This should be done

Table 1.7. Identity of Coalcon waste streams

Waste stream number	Flow rate (lb/hr)	pH	TSS (ppm)	Phenolics (ppm)	Soluble hydrocarbons (ppm)	Ammonia (ppm)	Hydrogen sulfide (ppm)	Trace ^a elements (ppm)	Other solubles (ppm)	Oil and grease (ppm)
3	530 ^b	2-5	100-4000	~0	~0	0.3-2.0	~0	C	SO ₄ ⁼ ~20,000 metal sulfides	~0
10	125,000	7-10	~0	100	<50	<50	<10	A	CN ⁻ <20 SCN ⁻ <50	trace
19	105,056	8-10	~0	10 ³ -10 ⁴	500-1,000	5,000-30,000	200-1000	B	CN ⁻ 10-200 SCN ⁻ 100-2000	<1%
23	12,331	5-9	~0	trace	trace	trace	~0	A	~0	trace
25	175,000	5-9	~0	200-6,000	100-600	<100	<25	B	CN ⁻ 6-120 SCN ⁻ 60-1200	<.6%
27	52,000	5-9	~0	<10	<5	trace	<1000	A	~0	trace
37	38,000	~7	~0	~0	~0	~0	~0	A	~0	~0
42	86,581	8-13	6,000-200,00	~0	~0	<200	~0	C	CN ⁻ , SCN ⁻ <1 ppm	~0
43	1,715	9-12	~0	0-1.2	0-1	<1-50	<.1	A	salts 10-500	~0
53	342,590	8-13	6,000-200,00	~0	NO	~0	~0	C	~0	~0
57	30,000	9-12	~0	0-1.2	0-1	<1-50	<.1	A	salts 10-500	~0
58	160,000	5-9	50-4,000	trace	trace	trace	~0	B	salts traces CN ⁻ and SCN ⁻	<50
59	358,000	7-10	<200	<0.1	trace	<1-80	<.1	A-B	salts ~2,400	trace
66	70,000	9-12	15,000-150,000	0	COD ~ 500-10,000	0	0	B-C	salts 2,000-12,000	0
67	265,000	~7	trace	0	~0	0	0	A	salts 4,000-18,000	0

^a A - little or no trace element content; B - possibly undesirable trace element content; C - significant trace element content.

^b Based on 90 day reserve storage pile.

from a best available technology standpoint as well as based on the relative hazards of the potential pollutants.

In addition to the Coalcon flowsheet study, existing federal effluent guidelines have been surveyed in order to anticipate effluent guidelines for future coal conversion plants. Table 1.8 summarizes the anticipated federal effluent guidelines and related process data.

Table 1.8. Summary of hydrocarbonization wastewater data

Effluent parameter	Total waste (lbs/day)*	Main sources	Hazardousness		Proposed Coalcon treatment	Existing treatment technology available	Anticipated regulations (lb effluent/10 ⁶ lb coal)
			Envir	Human			
pH			2	1	Neutralization	yes	6-9
TSS		Coal storage ash handling water treatment	2	1	Clarification sedimentation	yes	1-7
Phenolics	3600 - 25,200	Hydrocarbonization	3	3	Recycle to process	Expensive and unproven for coal wastes	0.01 - 0.15
Other soluble hydrocarbons (benzene, fatty acids)	1200 - 2500	Hydrocarbonization	2	2	Recycle to process	Expensive and unproven for coal wastes	BOD 2-15 COD 10-150
Oil and grease	~24,000	Leaks, plant washdown hydrocarbonization	2	2	Recycle to process	yes	.5 - 3
Dissolved gases (NH ₃ , H ₂ S, etc.)	Recovered for sales	Hydrocarbonization acid gas treatment			Stripping	yes	NH ₃ 0.4 - 2.5 S ²⁻ .01 - .06
Trace elements	Cr 480 Mn 1,608. Pb 858. Zn 13,500.	Ash handling coal storage	1-3	1-3	None	no	Cr 0.05 - .5 Mn .5 Pb .05 Zn .5
Other soluble contaminants	CN ⁻ 20-500 SCN ⁻ 200-5000	Hydrocarbonization acid gas treatment	3 2	3 2	Recycle to process	?	CN ⁻ 0.01 - 0.05 SCN ⁻ Unknown
PNA's	2-50	Hydrocarbonization leaks, spills	2	3	None	no	Unknown

* Based on coal feed 15,000 tons/day.
(3 x 10¹⁰ lbs/day)

¹ Fairly innocuous

² Undesirable.

³ Very hazardous.

1.5 References for Section 1

1. C. D. Scott et al., Experimental Engineering Sect. Semiannu. Prog. Rep. (Excluding Reactor Programs), March 1, 1975 to August 31, 1975, ORNL/TM-5291 (September 1976).
2. W. W. Pitt, Jr., R. L. Jolley, and C. D. Scott, "Determination of Trace Organics in Municipal Sewage Effluents and Natural Waters by High-Resolution Ion-Exchange Chromatography," Environ. Sci. Technol. 9, 1068-73 (1975).
3. R. L. Jolley, S. Katz, J. E. Mrochek, W. W. Pitt, and W. T. Rainey, "Analyzing Organics in Dilute Aqueous Solutions," Chem. Technol. May, 312-18 (1975).
4. C. D. Scott et al., Experimental Engineering Sect. Semiannu. Prog. Rep. (Excluding Reactor Programs), Sept. 1, 1975 to Feb. 29, 1976, ORNL/TM-5533 (in preparation).
5. W. W. Pitt, Jr., S. Katz, and L. H. Thacker, "A Rapid, Sensitive Method for the Determination of the Chemical Oxygen Demand of Polluted Waters," AIChE Symp. Ser. 129, 69 (1973).
6. R. L. Jolley, C. W. Gehrs, and W. W. Pitt, Jr., "Chlorination of Cooling Water: A Source of Chlorine-Containing Organic Compounds with Possible Environmental Significance," pp. 21-28 in Radioecology and Energy Resources, ed. by C. E. Cushing, Dowden, Hutchinson, and Ross Inc., Stroudsburg, Virginia, 1976.
7. R. L. Jolley, G. Jones, Jr., W. W. Pitt, Jr., and J. E. Thompson, "Chlorination of Organics in Cooling Waters and Process Effluents," p. 115-52 in Proceedings of the Conference on the Environmental Impact of Water Chlorination, CONF-751096, ed. by R. L. Jolley, National Technical Information Service, Springfield, Virginia, 1976.
8. R. L. Jolley, G. Jones, Jr., W. W. Pitt, Jr., and J. E. Thompson, "Determination of Chlorination Effects on Organic Constituents in Natural and Process Waters using High-Pressure Liquid Chromatography," in Identification and Analysis of Organic Pollutants in Water, ed. by L. H. Keith, Ann Arbor Science, Ann Arbor, Michigan, 1976.
9. R. B. Cumming, D. L. George, M. F. Walton, and J. B. Wlmhorst, "Mutations Produced by 5-Chlorouracil and 5-Bromouracil in *Escherichia coli*," pp. 135-36 in Biology Division Annu. Rep. for Period Ending June 30, 1975, ORNL-5072 (Nov. 17, 1975).
10. R. B. Cumming, B. C. Pall, M. F. Walton, and W. L. Russell, "Studies on Potential Effects of 5-Chlorouracil in Mammals," pp. 134-35 in Biology Division Annu. Rep. for Period Ending June 30, 1975, ORNL-5072 (Nov. 17, 1975).

11. R. B. Cumming, "The Potential for Increased Mutagenic Risk to the Human Population due to the Products of Water Chlorination," pp. 247-58 in The Proceedings of the Conference on the Environmental Impact of Water Chlorination, CONF-751096, ed. by R. L. Jolley, National Technical Information Service, Springfield, Virginia, 1976.
12. J. Marmur, "A Procedure for the Isolation of Deoxynucleic Acid from Microorganisms," J. Mol. Biol. 3, 208-18 (1961).
13. P. M. Williams and K. J. Robertson, "Halogenated Organic Compounds in Marine Organisms in the Thermal Plume Region of the San Onofre Nuclear Power Station," in Research on the Marine Food Chain Progress Report for the Period July 1974-June 1975, UCSD 10P20-202, Institute of Marine Resources, University of California.
14. E. T. Coles, Coalcon Commercial Plant Process Report, Contract No. E(49-18)-1736 (Aug. 15, 1975).
15. C. E. Jahnig and R. R. Bertrand, "Environmental Aspects of Coal Gasification," Chem. Engr. Prog., 72(8), 51 (August 1976).
16. E. M. Magee, C. E. Jahnig, and H. Shaw, Evaluation of Pollution Control in Fossil Fuel Conversion Processes, Gasification, Section 1, Kopper's Totzek Process, EPA-650/2-74-009a (January 1974).
17. C. D. Kalfaldelis and E. M. Magee, Evaluation of Pollution Control in Fossil Fuel Conversion Processes, Gasification, Section 2, SYNTHANE Process, EPA-650/2-74-009b (June 1974).
18. H. Shaw and E. M. Magee, Evaluation of Pollution Control in Fossil Fuel Conversion Processes, Gasification, Section 3, Lurgi Process, EPA-650/2-74-009c (July 1974).
19. C. E. Jahnig and E. M. Magee, Evaluation of Pollution Control in Fossil Fuel Conversion Processes, Gasification, Section 4, CO₂ Acceptor Process, EPA-650/2-009d (December 1974).
20. C. E. Jahnig, Evaluation of Pollution Control in Fossil Fuel Conversion Processes, Gasification, Section 5, BIGAS Process, EPA-650/2-74-009g (May 1975).
21. C. E. Jahnig, Evaluation of Pollution Control in Fossil Fuel Conversion Processes, Gasification Section 6, HYGAS Process, EPA-650/2-74-009h (August 1975).
22. C. E. Jahnig, Evaluation of Pollution Control in Fossil Fuel Conversion Processes, Gasification, Section 7, U-GAS Process, EPA-650/2-74-009i (September 1975).
23. C. E. Jahnig, Evaluation of Pollution Control in Fossil Fuel Conversion Processes, Gasification, Section 8, Winkler Process, EPA-650/2-74-009j (September 1975).

24. C. D. Kalfadelis and E. M. Magee, Evaluation of Pollution Control in Fossil Fuel Conversion Process, Liquefaction, Section 1, COED Process, EPA-650/2-74-009e (January 1975).
25. C. E. Jahnig, Evaluation of Pollution Control in Fossil Fuel Conversion Processes, Liquefaction, Section 2, SRC Process, EPA-650/2-74-009f (March 1975).
26. C. E. Jahnig, Evaluation of Pollution Control in Fossil Fuel Conversion Processes, Liquefaction, Section 3, H-COAL Process, EPA-650/2-74-009m (October 1975).
27. F. Glaser, A. Hershaft, and R. Shaw, Emissions from Processes Producing Clean Fuels, Report No. BA 9075-015 (March 1974).
28. J. F. Farnsworth, D. M. Mitsak, and J. F. Karmody, "Clean Environment with Koppers-Totzek Process," in Symposium Proceedings: Environmental Aspects of Fuel Conversion Technology, EPA-650/2-74-118 (May 1974).
29. J. A. Harshaw, H. D. Terzian, and L. J. Scotti, "Clean Fuels from Coal by the COED Process," in Symposium Proceedings: Environmental Aspects of Fuel Conversion Technology, EPA-650/2-74-118 (May 1974).
30. C. R. Gibson, C. A. Hammons, and D. S. Carneron, "Environmental Aspects of El Paso's Burnham I Coal Gasification Complex," in Symposium Proceedings: Environmental Aspects of Fuel Conversion Technology, EPA-650/2-74-118 (May 1974).
31. W. A. Parsons and R. A. Ashworth, "Coal Conversion Processes Wastewater Control," in Symposium Proceedings: Environmental Aspects of Fuel Conversion Technology, II, 650/2-76-149 (June 1976).

2. CENTRIFUGAL ANALYZER DEVELOPMENT

M. L. Bauer,* K. J. Beach,** W. D. Bostick, J. L. Fasching,*** R. K. Genung,
S. J. Hartmann,† W. F. Johnson,* N. E. Lee, J. E. Mrochek, C. D. Scott
J. B. Overton, and J. T. Walsh††

Development efforts during this reporting period were concentrated on the first prototype of the portable Centrifugal Fast Analyzer (CFA) and its temperature control system. Fabrication of the first production model of this new analyzer was 95% completed, and checkout of some of its sub-systems was performed. Preliminary testing of some aspects of the fast transfer of liquids and early monitoring of the transmittance of resulting solutions were performed with parallel-channel rotors on the first prototype.

Modification to the initial design of the multipurpose optical system (MOS) resulted in improved performance and diminished drift of the dark current. This latest design modification was incorporated in the retrofitting of the MOS located at the National Center for Toxicological Research (NCTR). The MOS on the CFA at the Clinical Center of the National Institutes of Health (NIH) which had been retrofitted previously was upgraded to include the latest modifications.

The study of new applications of CFA technology was continued in the general areas of clinical enzymology, immunology, hematology, chemistry, and environmental analysis. In addition, certain enzymes are being investigated for their potential as biochemical markers of cancer using the CFA. Evaluation of rotors preloaded with reagent tablets for four enzymic assays was completed.

*Instrument and Controls Division.

**Co-op Student.

***Visiting Scientist, University of Rhode Island, Kingston, R.I.

†Oak Ridge Associated Universities Summer Research Participant.

††Exceptional Student Program.

2.1 Multipurpose Optical System

General information on the design of a MOS suitable for retrofitting to the miniature CFA has been discussed previously.¹ Installation of the first retrofitted MOS on the NIH CFA was described in the last semiannual report.² Operation of this system revealed some problems which required some design changes. These changes were implemented in the MOS retrofitted to both the NCTR-2 CFA system and the NIH system. This section describes the details of the final design of the system.

2.1.1 Multipurpose optical system configuration

The NCTR-2 CFA, complete with all components of the retrofitted MOS, is illustrated in Fig. 2.1. The system incorporates two light sources; the original quartz-iodine-tungsten light located in a movable housing is shown rotated to the rear in Fig. 2.1. The second light source, an external 200-W xenon-mercury lamp with a small monochromator, directs its radiation into the rotor cuvetts by means of a quartz fiber optical bundle (see Fig. 2.1).

To enhance the light intensity impinging on the surface of the photomultiplier (PM) while at the same time minimizing optical alignment problems, a stationary two-lens assembly was designed. This assembly was designed to be used in conjunction with the secondary emission filter; however, it also serves a useful function in the transmission mode. The upper lens (see Fig. 2.2) focuses at the center of the cuvet and diminishes light losses due to inhomogeneities in rotor windows and baseplate. The lower lens (see Fig. 2.2) gathers the maximum amount of light from the upper lens and focuses it on the surface of the photomultiplier tube (PMT). Optical alignment is simplified since the lens system remains stationary

Fig. 2.1. The multipurpose optical system for the NCTR-2 Centrifugal Fast Analyzer.

Fig. 2.2. The fixed two-lens assembly of the multipurpose optical system. The filter holder is rotated between the upper and lower lens.

and the filter bar is rotated between the two lenses. A greater than tenfold increase in signal intensity is realized through use of the lens system for radiation > 340 nm (see Fig. 2.3).

It is necessary to increase the gain on the amplifier from the normal 10X in the automatic PM control mode to 1000X in order to operate the CFA in the fluorescence mode. This is accomplished by disabling the automatic PM voltage control with an external switch (see Fig. 2.4), thus enabling manual adjustment of the high voltage to the PMT. Modifications to the circuitry of printed circuit (PC) board A and associated connections to the new two-level switch (S-11A and S-11B) are indicated in Fig. 2.5 by Δ

Operational modes are changed from the normal automatic PM control for transmission measurements to manual control for use of the external xenon-mercury source (or vice versa) by readjusting the dark current to a small positive voltage (nominally 0.1 to 0.3 V). This adjustment is performed by means of an internal 10K potentiometer (R-8 on PC board A, see Fig. 2.5), which was made accessible to external adjustment (with a small screwdriver) by drilling a small hole in the top of the analyzer module (see Fig. 2.6).

It is necessary to operate the instrument only in the automatic PM control mode for transmission measurements to ensure that cuvet transmission or emission pulses are digitized correctly. Likewise, for any measurements made using the external xenon-mercury source, the instrument must be operated only in the manual PM control mode. This ensures that individual cuvet transmission pulses are properly positioned with their respective sample and hold pulses.

Fig. 2.3. Improvement in measurable signal afforded by use of the two-lens assembly on the miniature Centrifugal Fast Analyzer.

Fig. 2.4. Illustrated is the external switch which disables the automatic photomultiplier voltage control on the Centrifugal Fast Analyzer so that amplifier gain can be increased to 1000 for fluorescence measurements.

NO REPRESENTATION OR WARRANTY, EXPRESSED OR IMPLIED, IS MADE THAT THE USE OR DISCLOSURE OF ANY INFORMATION, APPARATUS, METHOD OR PROCESS DISCLOSED IN THESE DRAWINGS MAY NOT INFRINGE PRIVATE RIGHTS OF OTHERS. NO LIABILITY IS ASSUMED WITH RESPECT TO THE USE OF, OR FOR DAMAGES RESULTING FROM THE USE OF, ANY INFORMATION, APPARATUS, METHOD OR PROCESS DISCLOSED IN THESE DRAWINGS ARE BEING MADE AVAILABLE FOR INFORMATION TO BIDDER AND ARE NOT TO BE USED FOR OTHER PURPOSES, AND ARE TO BE RETURNED UPON REQUEST OF THE FORWARDING CONTRACTOR.	TOLERANCES UNLESS OTHERWISE SPECIFIED:	1	AS BUILT	A.J.F.	4-28-77			PORT. CENTRIFUGAL FAN ANALYZER					
	FRACTIONS ± _____	NO.	REVISIONS		DATE	APPD	APPD	PRINTED CIRCUIT BOARD					
	DECIMALS ± _____	DRAWN FARNER	DATE 4-28-77	SUBMITTED A.P. Edwards	DATE 6-20-77	APPROVED	DATE	No. Q2B62-125					
	ANGLES ± _____	DESIGNED T.A. ROCHER	DATE	APPROVED	DATE	APPROVED	DATE	SUBMITTED R.P. Edwards 7-5-77					
		CHECKED	DATE	APPROVED	DATE	APPROVED	DATE	ACCEPTED M.B. Bauer 6/27/77					
	SCALE:							REVISED J.E. Moore 7/5/77					
								E	12261	CD	028	D	REV.

Fig. 2.5. Modifications to circuit board A and the added two-level switch (S11-A, S11-B) to implement manual control of photomultiplier voltage for fluorescence measurements on the miniature Centrifugal Fast Analyzer are designated by Δ .

PHOTO 2239-76

Fig. 2.6. Illustrated is the hole drilled in the top panel of the Centrifugal Fast Analyzer to enable access to R-8 for adjustment of the dark current.

The utility of the MOS is dramatically pointed out in Fig. 2.7. Approximately a 50-fold improvement in sensitivity for the monitoring of reduced nicotinamide-adenine dinucleotide (NADH) is made possible by the use of fluorescence instead of transmission measurements. The fluorescence data shown in Fig. 2.7 were obtained with an excitation wavelength of 350 nm and a 420-nm "cut-on" filter for secondary emission on the miniature CFA.

2.2 Development of Portable Centrifugal Fast Analyzer

During this report period, a large amount of experimental effort has been expended on temperature control and dynamic measurement of cuvet-to-cuvet and within-cuvet temperature variations. Fabrication of the first production model of the portable CFA was 95% completed, and electronic checkout was begun. Experimental mixing studies were performed using two models of a parallel-chamber rotor.

2.2.1 Description of system

The first production model of the portable CFA is packaged somewhat differently from the prototype which was described previously.² The upper module (46 by 27.5 by 20 cm) contains all functional parts of the analyzer including the microprocessor, whereas the lower module (46 by 27.5 by 10 cm) contains the regulated power supplies with either ac or battery-containing modules available (see Fig. 2.8).

The analyzer requires 12, 24, and 28 V for operation; however, only 28 V is required for the motor. If a slightly lower motor speed is acceptable (3600 instead of 4000 rpm), the analyzer can be operated on two car batteries in series (24 V). This would enable operation for 9

ORNL DWG 76-1484

Fig. 2.7. Improvement in sensitivity afforded by monitoring fluorescence of NADH on the Centrifugal Fast Analyzer with multipurpose optical system.

Fig. 2.8. Power supplies for the portable Centrifugal Fast Analyzer; ac (left) and battery pack (right).

to 10 hr instead of operation for ~ 1 hr which can be obtained using the attached battery pack module.

Parameters describing the selected mode of data acquisition and the form of data analysis and output are entered into the analyzer via the first two thumbwheel switches on the upper panel (see Fig. 2.9). Table 2.1 lists the various modes of data acquisition together with their respective two-digit descriptors.

Data analysis and printout are controlled by entering a two-digit number on the first two thumbwheel switches after having completed a data acquisition. The tabular listings that are available are shown in Table 2.2. The slope or rate using either Program No. 04 or 06 are printed out as units (absorbance or intensity, respectively) per second. This may lead to a loss of significant figures since the results are printed out in decimal units. This situation is being corrected by the addition of another tabular listing which will enable multiplying the rate data (ODU/sec) by a four-digit factor for conversion to ODU/min or IU/liter (if an enzyme).

Errors in control parameters or in requests for data output will generate error messages consisting of a two-digit number. The error messages corresponding to these numbers are listed in Table 2.3. The first five error messages listed (01-08) are concerned with errors in acquisition control parameters, whereas the last nine messages (21-29) are concerned with data analysis and output.

2.2.2 Microprocessor control of the portable Centrifugal Fast Analyzer

Many functions of the portable CFA will be controlled by the microprocessor, as illustrated in Fig. 2.10. Some of the functional control

Fig. 2.9. Control panel for the portable Centrifugal Analyzer illustrating the 12 lever switches and their functions in data acquisition and analysis.

Table 2.1 Parameters for data acquisition program selection

Program No.	Mode of data acquisition	Rotor speed controlled by/at
00	Rotor calibration	Run potentiometer
01	Time-based ^a acquisition	Run potentiometer
02	Counts-based ^b acquisition	Run potentiometer
03	Time-based acquisition	500 rpm
04	Time-based acquisition	1000 rpm
05	Time-based acquisition	2000 rpm
06	Time-based acquisition	Accelerate potentiometer
07	Dual parameter ^c time-based acquisition	Accelerate potentiometer, run potentiometer
08	Counts-based acquisition	500 rpm
09	Counts-based acquisition	1000 rpm
10	Counts-based acquisition	2000 rpm
11	Counts-based acquisition	Accelerate potentiometer
12	Dual-parameter ^c counts-based acquisition	Accelerate potentiometer, run potentiometer

^aAnalyzer automatically accelerates the rotor for 150 msec, brakes for 100 msec, and accelerates to the run speed as set on the run potentiometer.

^bAnalyzer accelerates the rotor to the speed set on the run potentiometer and acquires data on selected rotor revolutions ($1 \leq 254$ revolutions between successive observations).

^cTwo different sets of parameters can be dialed into the analyzer: the first set defines data acquisition rate and number of observations over the initial portion of the reaction; the second set can be selected to obtain data at a much slower rate, thus taking measurements much later in the reaction when equilibrium conditions have been achieved.

Table 2.2. Data analysis and tabular listings available from the portable Centrifugal Fast Analyzer

Program No.	Message
00	Statistics for a rotor calibration
01	Absorbance - intensity
02	Differential absorbance
03	Differential intensity
04	Statistics on absorbance; slope (variation), intensity (variation), correlation coefficient
05	Statistics on absorbance, mean (variation)
06	Statistics on intensity; slope (variation), intensity (variation), correlation coefficient
07	Statistics on intensity, mean (variation)
08	Transmission counts ^a
09	Time table: If acquisition was time-based, a table of interval vs time is printed; if acquisition was counts-based, a table of two time values (1st and 16th cuvetts) for each observation is printed
10	Process control parameters
11	Concentration table ^b (factor x 1.0)
12	Concentration table (factor x 0.1)
13	Concentration table (factor x 0.01)

^aRaw data (not corrected by cuvet calibration factors) normalized to a full-scale voltage of 5 V and computed as $(\text{counts}/n)/16384 \times 5$ where n = number of revolutions averaged.

^b ΔA (operator-selected points) multiplied by a four-digit number entered by the operator.

Table 2.3. Error messages for the portable Centrifugal Fast Analyzer

Number	Message
01	Illegal program number
02	Illegal rotor calibration number (> 3)
06	Timing error
07	Revolutions between observations are out of range (> 256)
08	Run switch not in automatic
21	Transmission - dark current ≤ 0
22	Illegal cuvet number
23	Illegal final observation
24	Initial observation number $\geq$ final observation number
25	Illegal table request
26	Initial observation number equals final observation number
27	Negative concentration
28	Insufficient observations for statistical calculations (< 3)
29	No data acquired

Fig. 2.10. Functions of the portable Centrifugal Fast Analyzer under the control of the microcomputer.

is only a duplication of present capability on the miniature CFA. However, important new functions under control include filters and lamps, rotor temperature, rotor speed--clutch/brake, photomultiplier tube voltage, preamplifier gain, and microprocessor-mediated troubleshooting of the electronics. Brief descriptions of some of these new functional control aspects follow.

Rotor speed control. The electrical and mechanical aspects of rotor speed control are illustrated in Fig. 2.11. The rotor drive assembly consists of the motor/tachometer, inertial flywheel, clutch/brake, rotor baseplate, and multicuvet rotor. The clutch/brake device allows the rotor and baseplate to be disengaged from the inertial flywheel and the motor/tachometer. This feature is important in achieving rapid acceleration and braking of the rotor. The rotor remains motionless while the motor/tachometer and flywheel are accelerated to some preset run speed. When the clutch is engaged and the brake released, the rotor accelerates to 90% of its final run speed in 100 msec. During braking of the rotor, the brake is engaged and the clutch is released; this brings the rotor from run speed to a full stop in 100 msec. Signals to the motor and clutch/brake devices originate from computer output ports depicted in Fig. 2.11. A sample transfer-and-mixing sequence is described to clarify synchronization and control of rotor speed by the microprocessor.

A transfer-mixing sequence is initiated on the CFA to ensure thorough mixing of sample and reagent in the cuvet chambers of a sequential-addition rotor. It precedes any acquisition of transmission data during a time-based data acquisition. The sequence incorporates accelerate-brake-accelerate stages and is illustrated in Fig. 2.12. The rotor is accelerated from a stop to $\sim 88\%$ of run speed in the first 150 msec, then braked for

Fig. 2.11. Schematic representation of signal and data paths related to rotor speed control.

ORNL DWG 76-887R1

Fig. 2.12. Schematic rotor speed profile for time-based data acquisition on the portable Centrifugal Fast Analyzer.

120 msec, and again accelerated for 100 msec. Thus, 370 msec after the first movement of the rotor, the transfer-mixing sequence has been completed, and the acquisition of transmission data begins. This may be compared with an elapsed time of 5 to 7 sec between reaction initiation and the first acquisition of data on the miniature CFA.

When a transfer-mixing sequence is performed, the microprocessor checks initially that the brake is applied and the motor is off. This is accomplished by establishing the appropriate bit pattern on the 8-bit data bus. The processor selects the rotor port as the destination for the bit pattern by enabling $\overline{\text{OUTS}}$ (output device select) and by sending the rotor port code to the device decode logic (Fig. 2.11). This logic initiates a strobe signal $\overline{\text{RDS}}$ (rotor device select) to the rotor port which directs the port to read and latch the data bus information. The rotor port outputs three digital lines to the motor-speed decode circuitry whose function is to translate the bit pattern to an analog voltage for motor-speed regulation circuitry. The bit pattern decodes into motor off and seven motor run speeds. The rotor port also outputs two digital lines to two solid-state relays which control the clutch coil and the brake coil.

The next step in the transfer-mixing sequence is to accelerate the motor to a run speed while the rotor remains motionless. After a suitable delay, the rotor is accelerated by releasing the brake ($\overline{\text{BK}} = 1$)* and applying the clutch ($\overline{\text{CL}} = 0$). The computer clock is reset ($\overline{\text{CKR}} = 0$) and later read for the purpose of timing the 150-msec acceleration interval. The clock is read in 3 8-bit words, as strobed by $\overline{\text{CK1}}$, $\overline{\text{CK2}}$, and $\overline{\text{CK3}}$ clock-select signals. At the end of the 150-msec interval, the processor applies the brake and resets the clock. A 120-msec brake interval is now

*0 = false logic state; 1 = true logic state.

timed as the rotor decelerates from about 3500 rpm to a full stop. The brake interval is followed by a final acceleration interval of 100 msec (see Fig. 2.12). The acquisition of transmission data begins at the end of this acceleration interval or later if so directed by the operator-selected delay interval.

Photomultiplier tube voltage control. During the course of an acquisition, the microprocessor can control the anode voltage of the PMT. Figure 2.13 shows the feedback control loop used for this purpose. The microprocessor applies a controlling signal (v'_{HV}) to the variable high-voltage supply whose output is applied to the PMT anode. The parameter, v'_{HV} , originates from the bit pattern at the PM port which is translated into the analog signal, v_{HV} , by the DAC device (digital-to-analog converter). The parameter, v_{HV} , is buffered and level-shifted to yield v'_{HV} . The appropriate analog voltage is determined by examining the digitized transmission pulse for cuvet 1 (water blank). If this digital value indicates that the analog transmission pulse at the analog-to-digital converter (ADC) input is different from 4.9 V (ADC input range is 0 to 5 V), a new bit pattern is sent to the PM port.

Correct setting of the anode voltage on the PMT is necessary to ensure the maximum precision in acquired transmission data. If the PM voltage is too low, the digital range of the ADC is not completely utilized, and this causes loss of precision in the data. If the PM voltage is too high, it saturates the ADC, which renders the data meaningless.

Filters and lamps. The portable analyzer incorporates a MOS with an even greater flexibility than the MOS retrofitted to the miniature CFA.² The new system consists of two iodine-tungsten lamps and two sets of interference filters. One lamp and filter set (six interference filters)

Fig. 2.13. Schematic of automatic control of photomultiplier voltage by the microcomputer.

are positioned over the rotor to give the incident beam a 180° orientation to the PMT, as shown in Fig. 2.14(A). This set of filters is used for measurements of light transmission. Another lamp and filter set (ten interference filters) are positioned to introduce the incident beam at a 90° orientation relative to the PMT [Fig. 2.14(B)]. The filters in this set are positioned in pairs below and to the side of the rotor. These filters are used for measurements of fluorescence or light scattering.

The computer has the ability to select either lamp and any filter combination. This selection can occur during the course of an acquisition, thus yielding multiple wavelength data during a single analysis. Figure 2.14(C) functionally describes how filter selection is accomplished for the side filter set. The basis of the filter selection logic is a comparator and motor drive circuit. The comparator determines if the present filter is the filter requested by the processor. If the filter is not the one requested, the comparator will direct the filter motor to move the filter set until the requested filter is properly positioned. The current position of the filter is determined by the location of a binary-coded bar that is mechanically attached to the filter set [not shown in Fig. 2.14(A),(B)]. The binary signals from the bar are input to the comparator. Binary signals that designate the filter requested by the microprocessor originate from filter port 1 and are also input to the comparator.

When the filter set is in motion, the comparator circuit generates $BUSY = 1$. The processor reads $BUSY$ to determine if an excessive time (5 sec) is required to move the filter set. In practice, the need for extra time may occur because of mechanical restrictions, binding on the filter set, or electrical component failure. When $BUSY = 1$ for an

Fig. 2.14. Schematic illustration of (A) transmission light source and its filter bar, (B) side (90°) light source and its filter bar set, and (C) microcomputer control of the side filter bar.

excessive time, the processor generates an error signal to the comparator ($\overline{\text{ERR}} = 0$) which deenergizes the motor. The instrument operator is then informed of the malfunction on the printer device.

Other ancillary tasks performed by filter selection logic are lamp selection by the processor and the reading of the instrument's top-panel lamp switch and filter switch by the microprocessor. The logic shown in Fig. 2.14 is side filter and side lamp selection. The logic is duplicated in hardware for the top filter and top lamp selection.

Data acquisition and storage. The mechanical and electrical aspects of data acquisition on the portable CFA are very similar to those on the miniature CFA. Control logic for the microprocessor will be described since it is also involved. The rotation of the rotor baseplate by the stationary optical system results in a periodic series of light pulses which are detected by the PMT. Optical sensors which read a coded disc attached to the rotor shaft below the rotor baseplate (see Fig. 2.15) generate cuvet and rotor pulses. These pulses synchronize the ADC to the position of the rotor. The relationship of the cuvet and rotor pulses to the cuvet transmission pulses is illustrated schematically in Fig. 2.16. A rotor pulse occurs once every revolution between cuvet 17 and cuvet 1. This pulse triggers the ADC to digitize the dark current and also interrupts the microprocessor.

The microprocessor makes data acquisition and storage decisions synchronously with the occurrence of the rotor and cuvet pulses. These pulses are inputs to the vectored-interrupt logic whose function is to latch interrupt signals, generate vectored-interrupt instructions on the 8-bit data bus, and reset interrupt latches on microprocessor interrupt acknowledge (INTA) (see Fig. 2.15). When a rotor or cuvet interrupt

Fig. 2.16. Relationships between the transmission, rotor, and cuvet pulses on the portable Centrifugal Fast Analyzer.

occurs, the microprocessor executes a rotor service routine or a cuvet service routine, respectively. A flowchart of decisions made in the rotor service for a time-based acquisition is shown in Fig. 2.17. At a rotor speed of 4000 rpm, a 440- μ sec window exists between the rotor pulse and the No. 1 cuvet pulse (see Fig. 2.16). Execution of the rotor service routine, a sequence of 95 instructions, requires $\sim 380 \mu$ sec since the average execution time is 4 μ sec per instruction for the Intel 8080 microprocessor chip. Internal microprocessor registers are used as counters for observations recorded, revolutions recorded, and data- and time-storage addresses. On execution of an interrupt, these counters are incremented and tested. If an ADC reading for dark current needs to be stored, the ADC port is polled via ADC1 and ADC2 (see Fig. 2.15). Between successive observations, the microprocessor times the observation interval. During this time, rotor and cuvet interrupts are masked, thus allowing the rotor to revolve without interrupting the microprocessor. When the observation interval times out, the rotor interrupt is enabled, the interrupt latches are reset ($\overline{\text{IRES}} = 0$), the clock is reset ($\overline{\text{CKR}} = 0$), and the microprocessor enters a halt state (HLT) in anticipation of a rotor interrupt. When rotor interrupt occurs, the rotor service routine will enable the cuvet interrupt. The succeeding 17 cuvet interrupts will then direct the cuvet service routine to store time and transmission data as a new observation.

2.2.3 Temperature control

Heat transfer mechanisms and the temperature controller and control strategy in the portable or miniature CFA have been basically described in earlier progress reports.^{2,3} The changes (in the controller characteristics and the heat sink design) suggested in those reports have been

Fig. 2.17. Flowchart for the rotor service routine (time-based acquisition).

included in the most recently constructed portable CFA;* the evaluation of temperature control in the modified analyzer is in progress.

Evaluation of temperature control in the prototype portable CFA has been continued with the system shown schematically in Fig. 2.18. Typical responses from this system are shown in Fig. 2.19. The differences in dynamics shown by the pin and cuvet thermistors in Fig. 2.19 indicate the difficulties in tuning or controlling from the pin thermistor; it should be noted, however, that the dynamics shown by the pin thermistor qualitatively follow those measured by the cuvet thermistor.

The dynamics in cuvet fluid temperatures associated with acceleration of the rotor were investigated; temperatures of pretransferred fluids were monitored during a preheating period at 100 rpm and during the period following acceleration to higher rotor speeds. These results are shown in Fig. 2.20. Since the actual cuvet temperature variations were lower than those indicated by the pin thermistor (see Fig. 2.19), the results were initially viewed as qualitative. Mechanical limitations in the experimental system did not permit use of the cuvet thermistor at rotor speeds > 1000 rpm.

Figure 2.21 summarizes the initial problem areas in heat transfer and temperature control suggested by studies based on thermistor monitoring. While convective heat losses were assumed to be important primarily in long-term temperature control, it was suspected that uneven thermal contact could result in the rapid responses shown in Fig. 2.20. The efficiency of heat transfer referred to in Fig. 2.21 is related to the changes described in earlier reports.

Evaluation of temperature control in the prototype portable CFA was continued with the aid of optical thermometry. The technique employed

*Scheduled for delivery to the Environmental Sciences Division, ORNL.

ORNL DWG. 76-1451

Fig. 2.18. Schematic of temperature control on the portable Centrifugal Fast Analyzer.

Fig. 2.19. Comparison of liquid temperature within a cuvet with that monitored by the thermistor within an alignment pin. A relative measure of voltage to the heat pump is shown to illustrate the rate of controller response.

ORNL DWG 76-14443 AI

Fig. 2.20. Effect of acceleration rate on cuvet fluid temperatures.

Fig. 2.21. Problem areas in heat transfer and temperature control for the portable Centrifugal Fast Analyzer.

(recently presented in the literature)⁴ allowed monitoring of intercuvet and intracuvet temperature by use of two dye solutions; the optical properties of one dye are highly sensitive to temperature, whereas the optical properties of the other are insensitive to temperature. Cresol red (30 mg/liter) in Tris(hydroxymethyl)amino-methane buffer (0.1 M, pH 9.5) was used to construct an absorbance-vs-temperature calibration curve, while cresol red (10 mg/liter) in 1 M sodium hydroxide (absorbance was relatively temperature-insensitive) was used in the optical calibration of the cuvetts. Light transmission data, taken on the portable CFA by routine data acquisition procedures, were obtained during the periods of preheating, transfer and mixing, and reaction monitoring. These data, converted first to absorbance and then to temperature, allowed analysis of intercuvet and intracuvet temperature variations and, thus, temperature control for any period of interest.

Rotor calibrations. Two rotor calibrations were required before raw transmission data could be converted to absorbance data. The first calibration corrected for cuvet-to-cuvet surface differences (primarily reflectance phenomena), whereas the second calibration factor corrected for cuvet-to-cuvet pathlength differences. Both calibrations were applied to the following definition of relative transmittance T:

$$T = (\text{sample signal-dark current})/(\text{reference signal-dark current})$$

or

$$T_i = (C_i - C_D)/C_1 - C_D), \quad (1)$$

where C refers to "counts" as abstracted from photomultiplier output by the ADC, and the subscripts i, 1, and D refer to the ith (sample) cuvet, 1st (reference) cuvet, and the dark current, respectively. The transmittance T is defined as:

$$T = I_2/I_1 , \quad (2)$$

where I is intensity as used in Fig. 2.22. Using the following relationships with arbitrary constants R_1 and R_2 ,

$$I_1 = R_1 I_0, R_1 > 1 , \quad (3)$$

$$I_2 = R_2 I, R_2 < 1 . \quad (4)$$

Equation (2) can be rewritten as:

$$T = R_2 I / R_1 I_0 , \quad (5)$$

$$= f_s (I/I_0), \quad (6)$$

$$= f_s (T_{int}), \quad (7)$$

where f_s is a constant which is dependent on surface effects, and T_{int} is the internal transmittance associated with absorption phenomena.

Using Eq. (7), the transmittance of the i th cuvet relative to the reference cuvet (No. 1) can be expressed as:

$$\frac{T_1}{T_i} = \frac{(f_s)_1 (T_{int})_1}{(f_s)_i (T_{int})_i} , \quad (8)$$

$$= (F_s)_i (T_{int})_1 / (T_{int})_i , \quad (9)$$

where $(F_s)_i$ is referred to as the cuvet surface factor for the i th cuvet.

If all cuvetts contain only the same nonabsorbing solvent (e.g., distilled water), then:

$$(T_{int})_1 = (T_{int})_i = 1 , \quad (10)$$

ORNL DWG 76-17504

Fig. 2.22. Model for transmittance definition.

and the surface factors may be determined from Eq. (9) as:

$$(T)_1/(T)_i = (F_s)_i, \quad (11)$$

so that the following standardization is achieved:

$$(F_s)_i(T)_i = (T)_1. \quad (12)$$

With the first cuvet ($i = 1$), designated the reference cuvet, Eq. (1) gives

$T_i = 1$, and then

$$(F_s)_i = (T_i)^{-1}. \quad (13)$$

In the acquisition of data, the reference cuvet will contain a non-absorbing solvent, and the other cuvetts will contain absorbing samples.

In this case, $(T_{int})_1 = 1$, and Eq. (8) can be written as:

$$\frac{T_1}{T_i} = (F_s)_i \left(\frac{1}{T_{int}} \right)_i, \quad (14)$$

$$= (F_s)_i \left(\frac{I_o}{I} \right)_i. \quad (15)$$

Since (from Beer's Law)

$$I = I_o 10^{-\epsilon cb} = I_o e^{-kcb}, \quad (16)$$

where

ϵ = molar absorptivity;

c = molar concentration; and

b = pathlength, cm;

then Eq. (15) can be rewritten as:

$$\frac{T_1}{T_i} = (F_s)_i e^{(kcb)_i}. \quad (17)$$

If Eq. (17) is written for $i = 2$ and $i = 3$, the two resulting equations can be combined to give:

$$\frac{T_2(F_s)_2}{T_3(F_s)_3} = \frac{e^{(kbc)_3}}{e^{(kbc)_2}} = e^{(kbc)_3 - (kbc)_2} \quad (18)$$

or, in general, using cuvet No. 2 as a reference,

$$\frac{T_2(F_s)_2}{T_i(F_s)_i} = e^{(kbc)_i - (kbc)_2} \quad (19)$$

If cuvet Nos. 2 through 17 contain equal concentrations of the same absorbing fluid, Eq. (18) can be written as:

$$\frac{T_2(F_s)_2}{T_i(F_s)_i} = e^{kc(b_i - b_2)} \quad (20)$$

or

$$T_2(F_s)_2 = (F_p)_i T_i(F_s)_i \quad (21)$$

where a pathlength correction factor has been defined as:

$$(F_p)_i = e^{kc(b_i - b_2)} \quad (22)$$

An expansion of the exponential term clarifies the definition:

$$(F_p)_i = 1 + kc(b_i - b_2) + \frac{(kc)^2(b_i - b_2)^2}{2!} + \dots \quad (23)$$

As required, $(F_p)_i$ approaches 1 as b_i approaches the reference b_2 . The magnitude of $(F_p)_i$ can also be seen to depend on the physical properties of the absorbing species used in the measurement of pathlength factors.

In principle, Eqs. (21) through (23) can be used to correct for pathlength differences among cuvet Nos. 2 through 17. In practice, the pathlength factor, as defined by these equations, requires measurement of the actual

pathlengths and is therefore of limited value. However, a useful form is readily developed as follows:

$$A_2 = -\log[T_2(F_S)_2] = (kcb)_2 \quad , \quad (24)$$

$$A_3 = -\log[T_3(F_S)_3] = (kcb)_3 \quad , \quad (25)$$

$$A_i = -\log[T_i(F_S)_i] = (kcb)_i \quad ; \quad (26)$$

$$\frac{A_2}{A_i} = \frac{(kc)_2 b_2}{(kc)_i b_i} \quad (27)$$

$$\text{or } \frac{A_2}{A_i} = \frac{\log[T_2(F_S)_2]}{\log[T_i(F_S)_i]} \quad (28)$$

Since $(kc)_2 = (kc)_i$ for the same absorbing solution in all cuvet, Eq. (27) gives:

$$A_2 = (b_2/b_i) A_i \quad , \quad (29)$$

$$= (F_p)_i A_i \quad . \quad (30)$$

Similarly, Eq. (28) gives:

$$A_2 = \frac{\log[T_2(F_S)_2]}{\log[T_i(F_S)_i]} A_i \quad . \quad (31)$$

Comparing Eqs. (30) and (31) gives:

$$(F_p)_i = \frac{\log[T_2(F_S)_2]}{\log[T_i(F_S)_i]} \quad , \quad (32)$$

which depends only on transmittances corrected for surface effects. Equation (32) implies that all pathlength correction factors are based on the use of cuvet No. 2 as a standard reference for these factors. The $(F_p)_i$ value from Eq. (32) is then used to correct the A_i from Eq. (26) for pathlength effects;

$$(A_i)_{\text{correct}} = (F_p)_i \cdot A_i \quad (33)$$

Transmittance data corrected for both pathlength and surface effects can be calculated from basic definitions.

$$\begin{aligned} (T_i)_{\text{correct}} &= \log_{10}^{-1} (-A_i)_{\text{correct}} \\ &= \exp (-A_i/0.434)_{\text{correct}} \end{aligned} \quad (34)$$

The application of calibration factors presently available from the portable CFA is summarized as follows:

1. Transmittance data are obtained with cuvet Nos. 1 through 17 containing a nonabsorbing solvent (distilled water).
2. Surface factors are generated from Eq. (11) and automatically stored for a particular rotor. Since $(T)_i = 1$, $(F_s)_i = (T_i)^{-1}$.
3. Transmittance data are obtained with distilled water in cuvet No. 1 and the same absorbing solution in cuvet Nos. 2 through 17.
4. Surface-corrected transmittance data are automatically generated by the portable CFA using the results of Eq. (11).
5. Pathlength factors are generated from the surface-corrected transmittance data, $T_i(F_s)_i$, which the analyzer prints out by using Eq. (32).
6. For dynamic studies of temperature variation within a rotor, surface-corrected transmittance data are obtained with the solvent in cuvet No. 1 and solutions of the temperature-sensitive dye in cuvet Nos. 2 through 17.
7. Surface-corrected absorbance data are then generated from Eq. (26).
8. Absorbance data corrected for both pathlength and surface effects are generated from Eq. (33).
9. Transmittance data corrected for both pathlength and surface effects can be generated from Eq. (34).

10. Correlation of absorbance or transmittance data with other parameters can then be investigated.

In the prototype portable CFA, it should be reemphasized that the results of surface calibrations are stored as a $(F_s)_i$ vector and are automatically applied to the raw T_i ; the product $T_i \cdot (F_s)_i$ is available as an output of the prototype, but the individual cuvet factors, $(F_s)_i$, are not. Pathlength factors must be calculated by external means and applied as described above. This correction may be incorporated in the microprocessor of future instruments.

Temperature vs absorbance calibration. Data for the temperature vs absorbance calibration was taken in a Cary Model 14 spectrophotometer at 575 nm using a 1-cm jacketed cell. Temperature data were obtained with a calibrated thermistor inserted in the entrance to the cell; the temperature of the water circulating through the cell jacket was controlled with a Haake recirculating bath. A temperature vs absorbance calibration was obtained for both the temperature-insensitive and the temperature-sensitive dye solutions. Using temperature as the independent variable, the calibrations obtained were:

Temperature-insensitive dye

$$A = - 0.0025 (T, ^\circ\text{C}) + 1.0714 \quad (35)$$

Temperature-sensitive dye

$$A = - 0.0298 (T, ^\circ\text{C}) + 1.6980. \quad (36)$$

Equation (35) shows a sensitivity of 2.5 milliabsorbance units per $^\circ\text{C}$, whereas Eq. (36) shows a sensitivity of 30 milliabsorbance units per $^\circ\text{C}$. Data for these calibrations were obtained in the 25 to 35 $^\circ\text{C}$ range and were fitted by linear least-squares regression analysis. The calibrations, Eqs. (35) and (36), fit the data with an average error of 0.9%. Equation

(36) can be expressed with absorbance as the independent variable, thus giving the following useful equation:

$$T(^{\circ}\text{C}) = -33.476(A) + 56.954 \quad (37)$$

When the data were corrected to the 0.5-cm pathlength of the rotor cuvetts, the following equation resulted:

$$T(^{\circ}\text{C}) = -66.951(A) + 56.954 \quad (38)$$

Equation (38), which showed a sensitivity of 15 milliabsorbance units per $^{\circ}\text{C}$, was used in conjunction with Eq. (34) to perform temperature control studies on the portable CFA. Equation (38) fit the calibration data with an average error of 0.2°C . More calibration data will be taken to improve this fit for future work in temperature measurement, although it was adequate for measurement of temperature differences.

Results of intercuvet and intracuvet temperature monitoring. After calibrating surface and pathlength factors for an experimental rotor, it was loaded with buffer (0.1 M Tris, pH 7.5) in cuvet No. 1 and cresol red (30 mg/liter) in Tris buffer in cuvet Nos. 2 through 17. A sequential rotor was used, and 75 λ of solution was placed in both reagent and sample chambers; the solutions were transferred, the rotor was stopped, and the sample, reagent, and dynamic loading ports were taped closed. A 575-nm filter was positioned for monitoring, and the temperature control point was set at 30°C . Data were taken with the portable CFA MOS during the preheating period at 100 rpm, the temperature disturbance following an acceleration to 4000 rpm, and reaction-monitoring periods (10 to 15 min) under control at 30°C and 4000 rpm. The measurement periods are illustrated in Fig. 2.23.

Fig. 2.23. Long-term temperature control with pretransferred liquids (convective boundary conditions minimized).

Temperature variations from cuvet to cuvet during any measurement period were contained within a 0.85°C range; standard deviations for these cuvet-to-cuvet measurements were routinely 0.25°C . Ranges of intracuvet differences during the reaction monitoring period routinely appeared to be $< 0.05^{\circ}\text{C}$, with standard deviations routinely $< 0.02^{\circ}\text{C}$ over 10- to 15-min monitoring periods. The temperature-absorbance calibration was not sufficiently accurate to allow reliable monitoring of variations this small. Maximum fluctuations from the 30°C control point during the disturbance following the acceleration were found to be approximately $\pm 0.10^{\circ}\text{C}$.

In an effort to determine the source of apparent cuvet-to-cuvet temperature differences, thermal contact between the rotor and the heating plate was investigated. If uneven thermal contact existed, the rates of heating would vary from cuvet to cuvet, and the temperature history of cuvetts would differ. Figure 2.24, based on data taken during the period of greatest temperature change, demonstrated that heating rates did not vary significantly from cuvet to cuvet and, hence, that thermal contact was not a major problem during this period. The uniform difference between the highest and lowest cuvet temperatures (shown in Fig. 2.24) suggested a measurement problem in the system, probably associated with rotor calibration factors. The reproducibility of these factors is presently under investigation. Preliminary results indicate that run-to-run variations as well as day-to-day variations can be significant. Consideration is being given to the possibility that the reproducibility of optical alignment (dependent on the mechanical structure of the portable CFA) may limit the reproducibility of the pathlength factors and ultimately the limits of measurement with the instrument.

ORNL DWG 76-17505

Fig. 2.24. History of maximum and minimum cuvet temperatures during preheating period.

Comparison of temperature control on miniature and portable Centrifugal Fast Analyzers. A current comparison of temperature control on the prototype portable CFA with an operating miniature CFA was based on an enzymatic assay (glucose using hexokinase). The slopes of reactions progress curves for 15 replicate reactions were determined for assays run on each analyzer, and the standard deviation about the mean was computed. Reaction rate is reported to vary $\sim 7\%$ per degree centigrade around 30°C . The results are summarized in Table 2.4. These results indicate that temperature control on the prototype portable CFA compares very favorably with the presently accepted temperature control on the miniature CFA.

Future work. Future work will extend the use of optical thermometry. Improved calibrations will be developed, and temperature transients during transfer and mixing will be studied. Reproducibility of rotor calibration factors and optical alignment will be carefully examined, and the limits of temperature monitoring determined by variations in rotor calibrations will be defined. Future development work with the portable CFA will especially concentrate on temperature control under widely ambient temperature conditions.

Table 2.4. Comparison of a temperature-dependent enzymatic assay on the portable and miniature Centrifugal Fast Analyzers

	Portable	Miniature
Av slope ^a	0.3894	0.3761
Range	0.0145	0.0172
Standard deviation (SD)	0.0042	0.0043

^aBased on 15 individual replicates; difference in slope attributable to control at a nominal setpoint (30°C) on portable CFA and a calibrated setpoint (30°C) on miniature CFA.

2.2.4 The portable Centrifugal Analyzer - a precision spectrophotometer

One aspect of the CFA that has not been emphasized is its capability as a precision spectrophotometer. The ability to average many measurements enables precision measurements to be made on solutions differing by milliabsorbance units. Illustrated in Fig. 2.25 are measurements on dilutions of $K_2Cr_2O_7$ ranging from > 1 absorbance unit to < 1 milliabsorbance unit. An individual measurement illustrated in this figure is the mean of 99 measurements; each measurement is the average of 250 successive revolutions. Note that absorbances as low as 3 milliabsorbance units are measured with reasonable precision, whereas below 0.003 the precision decreases rather dramatically as might be anticipated. Changes in slope are undoubtedly due to errors introduced during successive dilutions since no attempt was made to calibrate the volumetric equipment.

2.2.5 Fast transfer and mixing

The ability to monitor fast kinetic reactions expands, by several orders of magnitude, the range of reaction rate constants that may be determined with accuracy. The regular sequential-addition rotor requires an accelerate-brake-accelerate sequence to ensure mixing and can be accomplished on the portable CFA within 0.4 sec. However, to exercise the full capability of the portable CFA for monitoring fast reactions within ~ 100 msec of their initiation, we cannot afford a braking step. A parallel-channel rotor (see Fig. 2.26) is being tested to determine whether acceleration to 4000 rpm will transfer and mix solutions of two different absorbances without a braking step.

As described previously,² blue dextran solutions of two different absorbances were used to determine the mixing characteristics of the parallel-channel rotor. Transfer of solution from the forward chamber

ORNL-DWG 76-1301A

Fig. 2.25. The portable Centrifugal Fast Analyzer employed as a precision double-beam spectrophotometer.

Fig. 2.26. Parallel-channel rotor.

is aided while transfer from the rear chamber is retarded by centrifugal force as shown schematically in Fig. 2.27. Results obtained for three successive transfer-and-mixing experiments are listed in Table 2.5 and depicted in Fig. 2.28. The data indicate that turbulence and incomplete transfer during the first rotor revolution cause rather large relative errors in mixing. The best apparent mixing was observed when the smaller volume (35 μ l) was loaded in the forward chamber, the larger volume (90 μ l) was placed in the back chamber, and data for the first revolution were deleted. The use of a 15- μ l volume in the back chamber, although yielding the smallest relative errors, is somewhat unrealistic in terms of actual operation because the small volume would not permit adequate dilution of the sample in this cavity.

2.2.6 Concept for documentation of mechanical components

The prototype of the portable CFA was developed from informal shop drawings which were frequently modified as the instrument was built and tested. Consequently, efforts are currently in progress to formally document the fabrication of the portable CFA. Such documentation will be required by any agency that intends to construct or maintain a portable analyzer for its own use.

Photography has been used to expedite documentation of mechanical components in order to save both engineering and drafting time. Using as a standard the first production model of the portable CFA, constructed after the prototype, each component of the disassembled instrument was photographed (multiple views were taken as needed for clarity). During reassembly of the instrument, subassembly photographs were made. A total of $\sim$ 150 photographs were taken and returned as 8- by 10-in. glossy prints and 4- 5-in. positives. The object on each glossy was cut out and

ORNL DWG. 76-1453

Fig. 2.27. Schematic for 1 cuvet of parallel-channel rotor.

Table 2.5. Relative errors observed in fast mixing^a for two homogeneous dye solutions of different concentrations

Volume in chamber (l)		Mean relative error ^b (%)		
Forward	Back	1-10 ^c	2-11 ^d	11-20 ^e
15	110	+11.25	+0.65	+0.38
25	100	10.92	0.85	0.59
35	90	7.36	0.23	-0.13
45	80	-0.01	-0.53	-0.84
55	70	-5.84	-0.64	-0.70
65	60	-4.75	-0.33	-0.46
75	50	-3.63	-1.08	-0.83
85	40	-6.70	-2.75	-1.25
95	30	-4.69	-1.36	-1.00
100	25	-3.45	-0.67	-0.71
105	20	-1.32	-0.43	-0.40
110	15	-0.85	-0.18	-0.13

^aUsing the parallel-channel rotor.

^bMean of three replicate experiments.

^cUsing mean value of absorbance measured over rotor revolutions 1 to 10; (observed-calculated)/calculated.

^dUsing mean value of absorbance measured over rotor revolutions 2 to 11.

^eUsing mean value of absorbance measured over rotor revolutions 11 to 20.

Fig. 2.28. Relatively complete mixing was observed when absorbance data for the first revolution were deleted.

mounted on 11- by 14-in. paper to permit room for easy layout of dimension lines and notes. Multiple views were placed on one sheet whenever possible. The 11- by 14-in. layouts were rephotographed and returned as mat prints, thus alleviating the draftsman's problem of lining on glossy prints.

The result of this procedure is illustrated in Fig. 2.29. Three sets of 75 photographs were made. The first set was retained by Engineering Coordination and Analysis Drafting Services for eventual use as a master; the second set was retained as a reference by the Advanced Analytical Systems Group. The third set is currently used by an ORNL instrument maker as working drawings in construction of the next portable CFA. (Construction schedules did not allow time for dimensioning parts on the photographed portable CFA.) As construction progresses, the instrument maker will obtain accurate parts dimensions and add these to his working drawings. As dimensions are standardized and approved, they will be given to Drafting Services for transposition to the master drawings. After the master mechanical drawings are completed, they will be organized by mechanical subsections and reproduced for formal documentation.

2.2.7 Inertial response in rotating systems

Well-known linear models have been developed that relate motor parameters to the time response of a dc motor driving an inertial load under given voltage and power supply conditions. These models can be used to predict the time responses of the ORNL miniature CFA. In general, the motor "transfer function" $G_m(s)$, which relates speed to input voltage, may be reduced to the following expression in the Laplace domain:¹

Fig. 2.29. Photographic views of a mechanical part of the portable Centrifugal Fast Analyzer with draftman's lines added.

$$\begin{aligned}
 G_m(s) &= \frac{\omega(s)}{V(s)} \\
 &= \frac{(1/K_e)}{(s\tau_m + 1)(s\tau_e + 1)}
 \end{aligned} \tag{39}$$

where

$\omega(s)$ = Laplace transform of speed;

$V(s)$ = Laplace transform of voltage;

K_e = voltage constant, volts/radian-sec.

τ_m = mechanical time constant of the motor, sec; and

τ_e = electrical time constant of the motor, sec.

The electrical time constant is a fixed parameter of the motor. The mechanical time constant depends on the inertial load added to the motor, as well as on the fixed motor parameters in the following manner:⁵

$$\tau_m = \frac{RJ}{K_e K_t} \tag{40}$$

where

R = radius of the disk, 1.75 in.;

J = inertial load, oz-in.-sec;² and

K_t = torque constant, oz-in./amp.

Both of the time constants represent the time for the rising exponentials to reach 63% of the final value. When Eq. (39) is solved in the time domain, one obtains:

$$\omega(t) = \frac{V}{K_e(\tau_m - \tau_e)} \left[-\tau_m e^{-t/\tau_m} + \tau_e e^{-t/\tau_e} \right] + V/K_e \tag{41}$$

Further, when we replace V/K_e by ω_{ss} (steady state rpm) and neglect the electrical time constant ($\tau_m \gg \tau_e$), Eq. (41) simplifies to the following expression:

$$\omega(t) = \omega_{ss}(1 - e^{-t/\tau_m}) . \quad (42)$$

If we define "response time," t_r , as the point at which we have obtained 90% final velocity, then

$$1 - e^{-t_r/\tau_m} = \frac{\omega(t_r)}{\omega_{ss}} = 0.90 \quad (43)$$

or

$$-t_r/\tau_m = \ln(0.1) = -\gamma . \quad (44)$$

Combining this result with Eq. (40), one obtains:

$$t_r = \gamma\tau_m = \frac{\gamma R J}{K_e K_t} , \quad (45)$$

or

$$t_r = \beta J , \quad (46)$$

where

$$\beta = \frac{\gamma R}{K_e K_t} .$$

Hence, t_r is a linear function of applied load. In Fig. 2.30, values of t_r as a function of J have been plotted for the Motomatic E-550 MG (the motor used in the miniature and portable analyzers). The inertial term has been converted to mass, where all the mass is in the form of a 3.50-in.-diam disk. The graph follows the linear equation:

$$t_r = (\beta R^2/2)M , \quad (47)$$

where

M = mass of the disk, g.

Fig. 2.30. Rise time as a function of inertial loads.

In the Motomatic system, the slope of this line is 0.0029 sec/g for a 90% response time. Thus, with each additional gram added in the form of a 3.50-in.-diam disk, the time required to achieve 90% of the final velocity would increase by 0.003 sec. This relationship is shown graphically in Fig. 2.30 and in tabular form in Table 2.6. Experimental verification of this relationship for the miniature analyzer was not obtained for the following reasons:

1. The inertial load of the miniature analyzer was not available.
2. The miniature analyzer is a completed design with no future alterations anticipated.

Analysis of the inertial response of the ORNL portable CFA currently under development was not subject to these constraints.

Table 2.6. Response times as a function of load

Mass (g)	Inertial load, J (oz-in.-sec ²)	63% rise time (sec)	90% rise time (sec)	99.9% rise time (sec)
150	0.0250	0.225	0.520	1.60
175	0.0285	0.257	0.59	1.78
200	0.0320	0.288	0.661	2.00
225	0.0355	0.320	0.735	2.22

Unlike the miniature CFA, however, the portable analyzer employs a clutch/brake to speed the time response. This introduction of a clutch/brake and flywheel into a system of motor and inertial load completely changes the analysis of time response. No longer are the properties of the motor of primary concern; the motor has reached a steady state speed

prior to the engagement of the clutch. Rather, the time response of the clutch between initial engagement and zero slip is of greatest importance.

A theory similar to the one previously discussed for the motor/load combination must be known to mathematically describe clutch engagement.

If the axial force pressing the clutch together is known and is assumed to be uniform across the contact surface, the resulting torque is given by:¹

$$T = (2/3)fF_a \frac{r_o^3 - r_i^3}{r_o^2 - r_i^2}, \quad (48)$$

where

T = torque capacity, lb_f-in.;

f = coefficient of friction;

F_a = axial force, lb_f;

r_o = outer radius of clutch plate, in.;

r_i = inner radius of clutch plate, in.

When the definition of torque is recalled, we may rewrite Eq. (48) in the following manner:

$$\alpha = d\omega/dt, \\ = (1/J_{load})(2/3)fF_a \left[\frac{r_o^3 - r_i^3}{r_o^2 - r_i^2} \right]. \quad (49)$$

Thus, in order to obtain angular velocity as a function of time, one must know the axial force as a function of time. If the axial force is constant, acceleration will also be constant. If the driving mechanism attempts to transmit more torque than that given by the right side of Eq. (48), slippage will result. In other words, the axial force determines the maximum torque that a clutch may transmit and, for a given inertial load, the maximum acceleration of that load.

For electromagnetic clutches of the type used in the portable CFA, the axial force may be approximated in the following manner. When the energy balance in separating the magnets a small distance is considered, as in Eq. (42), we obtain:

Mechanical input + electrical input = magnetic energy storage in air gap + heat,

or

$$F_a dx + i^2 R dt = (1/2)(B^2/u_0) A dx + i^2 R dt \quad , \quad (50)$$

where

dx = small distance of plate separation;

i = current;

R = coil resistance;

dt - small time element;

B = magnetic field strength;

u_0 = magnetic permeability of air;

A = area of clutch interface.

This balance assumes either a uniform F_a or one derived by averaging the values across the plate. Solving for F_a , we obtain:

$$F_a = \frac{B^2 A}{2u_0} \quad . \quad (51)$$

If we assume a current sufficient to saturate the electromagnet, then B is constant, and therefore F_a is constant. Thus, for an electromagnet at saturation we would expect a constant axial force and constant angular acceleration predicted by Eqs. (49) and (51). This explains the nature of Fig. 2.31, a generalized clutch-response curve which applies to the

Fig. 2.31. Generalized clutch-brake response (reproduced from Formsprag-Simplatrol).

Simplatrol clutch/brake used in the portable CFA. After an initial lag period, the clutch provides constant angular acceleration proportional to the maximum axial force. When the angular speed of the load reaches that of the driving motor and flywheel, no further torque is transmitted, and the speed becomes constant.

One other result was obtained from Eq. (49). If F_a is constant, the acceleration (α) is inversely proportional to the load. Thus,

$$\alpha_1/\alpha_2 = J_2/J_1$$

or

$$\alpha_2 = (J_1/J_2)\alpha_1 \quad (52)$$

Hence, doubling the inertial load would result in halving the rate of acceleration. Rewriting this expression in a different way:

$$d\omega/dt = \delta/J \quad (53)$$

where

$$\delta = (2/3)fF_a \left[\frac{r_o^3 - r_i^3}{r_o^2 - r_i^2} \right]$$

If Eq. (53) is integrated with the boundary condition $\omega(0) = 0$, then

$$\omega(t) = (\delta/J)t \quad (54)$$

or

$$t = \frac{\omega(t)}{\delta} J \quad (55)$$

Thus, it is clear that the time t_r to arrive at any given velocity should be a linear function of inertial load. If we again define t_r as response time or time required to reach 90% of terminal velocity, we can check Eq. (49) by monitoring the speed experimentally.

The portable CFA is well equipped to produce an experimental history of speed. It contains an internal clock which reads to an accuracy of 0.1 msec. Further, the unit's microprocessor contains a standard program which records the time that cuvet Nos. 1 and 16 pass. Since the spacing between these cuvetts is 2/17 of a revolution (0.739 radians), the approximation that velocity is constant during this small portion of a revolution is reasonable, and the velocity can be obtained once each revolution:

$$\omega_{av,n} = \left[\frac{t_{1,n} - t_{16,n-1}}{0.739} \right]^{-1}, \quad (56)$$

where

$\omega_{av,n}$ = av velocity at the nth revolution;

$t_{1,n}$ = time that cuvet 1 passes in the nth revolution;

$t_{16,n-1}$ = time that cuvet 16 passes in the (n-1)th revolution.

In the experiments actually conducted, the program described above was executed for various rotor loadings in order to determine the change in response to increasing loads. The analyzer's motor/flywheel was brought up to 4000 rpm, and the clutch was then engaged. Data were taken for ~ 0.5 sec in each case. The response time in each case was recorded as the time required to reach 90% of final velocity. Figure 2.32 presents a typical curve of response time obtained in this fashion. The response times are also plotted vs load (rotor mass) in Fig. 2.33. From these figures, it is clear that a linear relationship exists between rotor mass and response time. The slope of the line indicates that response time will be slowed by ~ 0.54 msec for each gram of mass added to the inertial load (if the mass is added in the form of a 3.50-in.-diam disk). The linearity in Figs. 2.32 and 2.33 confirms the use of

Fig. 2.32. Acceleration curve for portable Centrifugal Fast Analyzer.

Fig. 2.33. Response time as a function of load.

Eq. (49). Thus, when an electromagnetic clutch is used, acceleration and response time were shown to decrease linearly with increased inertial load as predicted by the model employed.

2.3 Applications

One of the more interesting aspects of implementing new technological developments is the participation in research on their application to practical problems. This section is devoted to new applications of the CFA in the areas of biomedical and environmental analysis.

2.3.1 Development of Centrifugal Fast Analyzer rotors preloaded with reagent

Stability studies are continuing on CFA rotors preloaded with reagents. Rotors containing reagent tablets for the assay of SGOT (serum glutamyl oxalotransaminase), AP (alkaline phosphatase), and LDH (lactate dehydrogenase) were evaluated against freshly reconstituted tablets and fresh reagents. Assays were performed to test the stability of reagent tablets in rotors which were stored in a refrigerated desiccator during a 10-week evaluation period.

Evaluation of SGOT reagent tablets. The specifications for the SGOT reagent tablets (Smith-Kline) were reproduced previously.² Results obtained during the 10-week stability tests demonstrated good agreement between activities using tablets sealed in rotors (reconstituted in situ) compared with activities from freshly opened vials of tablets (see Tables 2.7 and 2.8).^{*} The relative variation in activities on a day-to-day basis was larger by approximately a factor of 2 for in situ reconstitution compared with fresh tablet reconstitution (see Table 2.8). However, the former represents inter-tablet variation and is expected to be higher than the inter-vial variation which the relative standard deviation (RSD)

^{*}Individual tablets sealed in the cuvetts of rotors at the beginning of the ten-week evaluation will generate statistical data on inter-tablet variation. Bottles containing 20 tablets are opened and immediately reconstituted at the time of use (freshly opened) and will generate statistical data on inter-vial variation.

Table 2.7. Evaluation of preloaded rotors and fresh tablets versus standard reagent kits for the enzymatic assay of serum glutamyl oxalotransaminase

STANDARD #1

EXP. #	S.K. IN SITU			S.K. FRESH			CALBIOCHEM		
	MEAN	SD	RSD	MEAN	SD	RSD	MEAN	SD	RSD
1	17.9	0.6	3.4	16.0	0.4	2.6	14.7	0.3	1.9
2	23.5	1.1	5.4	19.3	1.2	6.3	12.5	0.4	3.1
3	19.3	1.1	5.8	17.1	0.4	2.6	14.1	0.6	3.9
4	18.4	1.3	7.1	15.6	1.0	6.4	12.1	0.1	1.1
5	17.3	0.6	3.5	18.0	0.6	3.2	14.2	0.6	4.0
6	18.1	0.4	2.3	16.6	3.4	2.1	14.0	0.4	2.5
7	12.2	2.6	23.9	15.2	0.9	6.0	15.0	0.4	2.5
8	17.6	0.3	1.6	17.5	0.3	1.5	15.4	1.2	7.8
9	17.9	2.8	15.8	17.0	0.4	2.0	12.9	0.7	5.6
10	16.0	0.7	4.4	17.9	3.6	3.3	13.2	0.5	3.7

STANDARD #2

EXP. #	S.K. IN SITU			S.K. FRESH			CALBIOCHEM		
	MEAN	SD	RSD	MEAN	SD	RSD	MEAN	SD	RSD
1	27.3	0.3	1.0	29.6	1.0	3.4	26.2	0.6	2.1
2	31.4	3.4	1.2	31.1	0.1	0.3	23.7	0.6	2.3
3	28.6	0.3	1.2	28.9	0.3	3.9	24.5	0.4	1.4
4	28.5	0.4	1.2	27.8	0.4	1.4	22.6	0.3	1.3
5	29.6	1.3	4.2	29.7	0.6	1.8	23.7	1.4	5.7
6	29.5	0.9	3.0	29.5	0.3	3.9	23.2	0.2	0.8
7	20.0	1.6	8.2	26.1	0.3	1.2	22.9	0.2	0.9
8	28.7	3.5	1.6	30.1	0.3	0.8	26.7	1.1	4.1
9	28.9	0.9	3.1	29.2	0.4	1.4	23.3	0.5	2.0
10	28.5	1.0	3.6	29.5	3.3	0.8	24.4	0.6	2.3

STANDARD #3

EXP. #	S.K. IN SITU			S.K. FRESH			CALBIOCHEM		
	MEAN	SD	RSD	MEAN	SD	RSD	MEAN	SD	RSD
1	53.3	2.2	4.1	52.4	0.4	0.8	46.3	0.7	1.4
2	56.0	2.8	5.0	56.5	0.8	1.4	43.9	0.2	0.4
3	50.2	1.4	2.9	51.4	0.9	1.7	43.9	0.7	1.7
4	50.4	0.9	1.8	51.8	0.1	0.2	43.7	0.5	1.1
5	46.9	1.3	2.8	52.3	0.7	1.4	43.7	1.1	2.4
6	57.5	1.1	1.9	50.8	0.3	0.6	47.2	0.6	1.2
7	46.2	4.7	10.1	52.8	0.6	1.2	44.8	0.6	1.2
8	50.4	0.4	0.8	52.9	0.2	0.4	46.5	1.2	2.5
9	50.6	0.2	0.5	50.4	1.0	1.9	42.0	0.5	1.1
10	50.5	0.3	0.7	51.9	0.3	0.5	43.8	0.2	0.6

STANDARD #4

EXP. #	S.K. IN SITU			S.K. FRESH			CALBIOCHEM		
	MEAN	SD	RSD	MEAN	SD	RSD	MEAN	SD	RSD
1	122.2	2.0	1.7	119.3	0.7	0.6	104.9	1.7	1.7
2	133.2	2.1	1.6	132.6	1.6	1.2	110.7	0.4	0.4
3	120.7	1.0	0.8	124.4	0.7	0.6	109.5	1.2	1.1
4	121.6	0.5	0.4	123.3	1.2	1.0	109.1	0.6	0.5
5	117.8	1.6	1.3	123.0	1.6	1.3	107.1	0.9	0.9
6	135.6	2.1	1.6	116.7	0.3	0.2	113.3	0.4	0.4
7	114.5	3.5	3.1	122.0	1.9	1.5	108.6	1.6	1.5
8	123.0	2.7	2.2	123.0	0.8	0.7	111.6	1.1	1.0
9	111.5	1.1	0.9	117.9	1.0	0.8	100.5	1.8	1.7
10	117.2	3.5	0.4	116.5	0.3	0.2	104.7	1.7	1.7

STANDARD #5

EXP. #	S.K. IN SITU			S.K. FRESH			CALBIOCHEM		
	MEAN	SD	RSD	MEAN	SD	RSD	MEAN	SD	RSD
1	244.2	4.5	1.9	234.7	1.5	0.7	208.4	2.2	1.1
2	257.8	2.1	0.8	248.7	0.6	0.2	238.7	10.0	4.2
3	235.9	1.7	0.7	237.2	1.4	0.6	210.4	1.6	0.7
4	232.1	3.0	1.3	226.7	3.4	1.5	230.3	1.1	0.5
5	228.7	1.0	0.5	237.2	2.3	1.0	209.3	2.7	1.3
6	240.0	2.9	1.2	237.1	2.0	0.8	238.6	3.1	1.5
7	236.1	8.9	3.8	233.7	1.6	0.7	214.7	1.8	0.9
8	243.9	2.6	1.1	241.7	1.6	0.7	222.6	5.1	2.3
9	240.1	4.0	1.7	245.9	0.9	0.4	202.7	4.3	2.1
10	238.0	0.6	0.2	234.3	2.5	1.1	215.0	2.4	1.1*

for reconstituted fresh tablets represents. Note that both within-day and day-to-day precision for fresh tablets were slightly better than that for the commercial reagent kit (see Table 2.9). Measurement of absorbances for reagents only (Table 2.10) indicated that inter-tablet and inter-vial variation for the tablets was quite low; and, hence, that the tablets contained a precise amount of NADH. In summary, the data indicate that the reagent tablets are stable and yield acceptable measurements of SGOT activity for at least 10 weeks after being sealed in rotors.

Table 2.8. Day-to-day variation - SGOT reagent tablet stability test

Std. No.	S.K. in situ			S.K. fresh			Calbiochem.		
	Mean	SD	RSD	Mean	SD	RSD	Mean	SD	RSD
1	17.48	2.41	13.80	17.01	1.32	7.73	13.79	1.15	8.31
2	28.27	3.03	10.72	29.14	1.36	4.68	24.09	1.43	5.93
3	51.18	3.81	7.44	52.32	1.70	3.24	44.57	1.65	3.69
4	121.52	7.71	6.34	121.85	4.70	3.86	107.99	3.81	3.53
5	239.90	8.45	3.52	237.69	6.34	2.67	213.95	10.43	4.87

Table 2.9. Comparison of precision data with three SGOT reagent preparations

Reagent	Average within-day (% RSD)	Average day-to-day (% RSD)
SKI tablets (in situ) ^a	3.13	8.36
SKI tablets (fresh) ^b	1.49	4.44
Calbiochem ^c	1.98	5.27

^aSmith Kline Instruments, Inc.; sealed in rotors; tablets reconstituted individually at time of use.

^bSealed 20-tablet vials; vials opened and entire contents reconstituted at time of use.

^cCommercial reagent kits.

Table 2.10. Day-to-day variation - SGOT reagent tablet blank absorbances

Exp. No.	S.K. in situ	S.K. fresh	Calbiochem.
1	0.6486	0.5189	0.5000
2	0.6414	0.5252	0.6652
3	0.6491	0.5112	0.6095
4	0.6474	0.5121	0.6867
5	0.6072	0.5036	0.6629
6	0.6922	0.4547	0.6068
7	0.6931	0.5162	0.4515
8	0.6613	0.5524	0.5095
9	0.6272	0.5364	0.4717
10	0.6051	0.5506	0.5255
*Mean	0.6473	0.5181	0.5689
SD	0.0301	0.0278	0.0872
RSD, %	4.6	5.4	15.3

Evaluation of alkaline phosphatase reagent tablets. The reagent tablets for the kinetic assay of AP did not include the buffer. The buffer, diethanolamine (DEA), a separate constituent, was added during the in situ dissolution process. The manufacturer's specification and performance data for the tablets (lot No. 30-4-47) and DEA buffer (lot No. 30-4-58) are tabulated below:

1. pH 10.0 $\pm$ 0.1	1. pH of buffer = 9.95 $\pm$ 0.03 n = 6
pH of tab + buffer = 9.90
2. Tablet weight 10.0 $\pm$ 0.5 mg	2. 10.17 mg $\pm$ 0.28 n = 14
cv (coefficient of variation) = 2.8%
3. Dissolution time	3. 34 sec
4. Activity at 30°C:
Eskalab Abnormal No. 4448
range (270-350)
Hyland Q Pak
Multi Enzyme "C"
Multi Enzyme "A"	4. at 30°C 311 IU ^a $\pm$ 10.4
n = 6
"C" at 30°C 144 IU $\pm$ 9 n = 8 cv = 6.3%
"A" 72 IU $\pm$ 4 n = 7
cv = 9.7%

^aTo obtain this high value, parameters used were equivalent to a 127.5- μ l reagent: 2.5- μ l sample. Reagent rate limit is Δ max of 0.1/min or $\sim$ 75 IU with 120 μ l: 10- μ l parameter.

The measured activity for AP in human serum is markedly dependent on the medium in which the reaction takes place. Some buffers support much higher levels of enzymatic activity because of transphosphorylation; for example, the net transfer of phosphate from the substrate to the hydroxyl group of the buffer is a more rapid reaction than transfer to water (hydrolysis).⁶ Diethanolamine, the buffer used to formulate the reagent tablets, has transphosphorylation activity, whereas the buffer used in the commercial kits does not. Thus, AP activities measured using the reagent tablets were higher than those measured with the commercial reagent kits (Calbiochem).

A problem encountered during the early weeks of the 10-week stability study on these tablets was the relatively large variations in the measured activity for the fresh tablets (especially weeks 2 and 4, Table 2.11). This was accompanied by elevated absorbances for reagent blanks (see Table 2.12) and apparent high reagent blank activities (see Table 2.13).

Consultation with the reagent tablet manufacturer suggested that differences in the quality of the distilled water used in reagent reconstitution could be the cause of varying reagent blank activity. The final 4 weeks of the stability tests were performed with a higher quality of distilled water which had been freshly drawn just prior to performing the test assays. Activities and absorbances of the blank seemed to be more uniform for the final week of the test, and there were no excursions from expected activity measurements on the standard serum. It seems apparent from Table 2.12 that if water quality was a problem, the commercial reagent kit was apparently a less sensitive means of detecting this problem (compare blank absorbances for week 2).

Table 2.11. Evaluation of preloaded rotors and fresh tablets versus standard reagent kits for the enzymatic assay of serum alkaline phosphatase

STANDARD #1

EXP. #	S.K. IN SITU			S.K. FRESH			CALBIOCHEM		
	MEAN	SD	RSD	MEAN	SD	RSD	MEAN	SD	RSD
1	16.6	1.1	6.4	20.9	0.4	1.7	14.5	0.2	1.1
2	19.6	0.6	3.1	34.4	0.8	2.3	14.1	0.2	1.1
3	16.2	0.4	2.4	23.3	0.5	2.3	16.6	0.1	0.3
4	18.4	0.6	3.2	33.4	0.3	0.9	15.6	0.1	0.3
5	16.4	0.9	5.2	17.9	0.6	3.2	15.9	1.2	7.2
6	16.0	0.6	3.7	20.2	0.2	1.1	13.5	0.2	1.4
7	16.4	0.5	3.2	18.1	0.2	0.9	14.6	0.1	0.6
8	16.7	0.8	4.8	18.6	0.3	1.8	13.9	0.1	0.5
9	17.2	0.3	1.8	21.2	0.1	0.5	13.3	0.1	0.4
10	14.5	0.1	0.7	19.2	0.2	1.2	14.1	0.1	0.3

STANDARD #2

EXP. #	S.K. IN SITU			S.K. FRESH			CALBIOCHEM		
	MEAN	SD	RSD	MEAN	SD	RSD	MEAN	SD	RSD
1	42.1	2.1	5.0	44.9	0.3	0.7	31.3	0.1	0.3
2	40.1	3.7	9.3	55.3	0.8	1.4	31.0	0.3	1.0
3	39.9	2.4	5.9	46.3	1.1	2.3	33.2	0.2	0.4
4	44.3	1.8	4.0	60.2	0.4	0.7	32.9	0.3	0.8
5	35.2	3.6	10.2	39.4	0.9	2.4	34.1	1.8	5.4
6	41.3	1.1	2.6	47.0	0.7	1.5	29.8	0.3	1.0
7	38.5	0.8	2.1	43.8	0.5	1.1	31.3	0.1	0.2
8	41.3	0.3	0.7	43.3	0.2	0.4	31.5	0.1	0.4
9	41.4	0.1	0.1	45.4	0.2	0.4	29.0	0.1	0.4
10	39.3	0.4	1.1	43.8	0.6	1.4	30.4	0.1	0.5

STANDARD #3

EXP. #	S.K. IN SITU			S.K. FRESH			CALBIOCHEM		
	MEAN	SD	RSD	MEAN	SD	RSD	MEAN	SD	RSD
1	79.9	3.8	4.8	79.9	0.4	0.5	54.9	0.4	0.7
2	77.6	2.0	2.6	86.6	1.2	1.4	56.2	0.5	0.9
3	79.2	3.3	4.2	81.7	0.7	0.9	57.5	0.6	1.1
4	87.0	2.2	2.5	95.1	0.7	0.8	57.8	0.5	0.9
5	65.0	2.2	3.3	70.7	1.0	1.4	55.7	0.6	1.1
6	80.0	2.0	2.5	86.4	0.6	0.7	54.5	0.4	0.7
7	74.0	1.3	1.8	82.5	2.2	2.6	56.0	0.9	1.5
8	75.8	1.0	1.3	78.6	0.3	0.4	56.3	0.4	0.8
9	77.7	2.2	2.8	79.7	0.1	0.2	52.8	0.6	1.0
10	75.8	1.3	1.7	78.4	0.4	0.5	53.7	0.7	1.3

STANDARD #4

EXP. #	S.K. IN SITU			S.K. FRESH			CALBIOCHEM		
	MEAN	SD	RSD	MEAN	SD	RSD	MEAN	SD	RSD
1	112.7	2.2	2.0	109.4	0.6	0.6	77.8	0.1	0.1
2	97.8	2.6	2.7	108.5	1.5	1.4	75.3	0.1	0.1
3	102.7	2.5	2.4	106.8	1.6	1.5	73.9	0.0	0.0
4	110.7	1.7	1.5	120.1	1.0	0.8	74.5	0.1	0.1
5	114.7	1.4	1.2	120.9	1.0	0.8	92.4	0.3	0.3
6	105.8	2.1	2.0	115.7	1.2	1.0	71.9	0.8	1.1
7	99.8	1.0	1.0	110.3	0.8	0.8	73.0	0.1	0.1
8	94.9	1.8	1.9	101.6	1.1	1.0	74.4	0.3	0.4
9	102.0	1.0	0.9	102.4	0.4	0.4	69.8	0.3	0.4
10	106.9	0.7	0.6	108.1	1.0	1.0	72.8	0.5	0.7

STANDARD #5

EXP. #	S.K. IN SITU			S.K. FRESH			CALBIOCHEM		
	MEAN	SD	RSD	MEAN	SD	RSD	MEAN	SD	RSD
1	140.5	4.6	3.3	139.5	0.3	0.2	102.3	0.3	0.3
2	138.8	4.3	3.1	141.8	1.2	0.8	108.1	0.5	0.4
3	146.0	4.2	2.9	149.8	3.9	2.6	106.0	0.4	0.3
4	156.7	1.2	0.8	166.3	1.9	1.1	108.0	0.8	0.7
5	127.1	1.5	1.2	126.7	0.8	0.6	104.0	0.9	0.9
6	146.8	1.8	1.2	160.7	0.8	0.5	102.1	0.5	0.5
7	139.7	1.1	0.8	160.9	1.0	0.6	105.1	0.4	0.4
8	144.1	9.1	6.3	143.1	0.6	0.4	108.7	1.0	0.9
9	144.1	1.2	0.8	143.0	0.1	0.1	101.4	0.4	0.4
10	145.4	5.6	3.9	145.7	1.7	1.2	100.6	0.2	0.2

Table 2.12. Day-to-day variation - alkaline phosphatase reagent tablet blank absorbances

Exp. No.	S.K. in situ	S.K. fresh	Calbiochem.
1	0.4092	0.3421	0.2885
2	0.7111	0.9624	0.2928
3	0.4381	0.3899	0.2875
4	0.4128	0.5148	0.2988
5	0.3761	0.3300	0.2626
6	0.4999	0.3302	0.2714
7	0.4165	0.3323	0.2694
8	0.4780	0.3228	0.2738
9	0.3805	0.3436	0.2731
10	0.3899	0.3166	0.2456
Mean	0.4512	0.4185	0.2764
SD	0.0998	0.2000	0.0159
RSD, %	22.1	47.8	5.8
<u>Without No. 2</u>			
Mean	0.4223	0.3580	0.2745
SD	0.0427	0.0625	0.0157
RSD, %	10.1	17.5	5.7

Table 2.13. Within-day variation - alkaline phosphatase reagent tablet blank activity

Exp. No.	S.K. in situ	S.K. fresh	Calbiochem.
1	0.6316	4.0054	2.6108
2	7.1727	16.2682	1.6540
3	1.3579	6.1003	4.6955
4	2.1391	14.0446	2.5616
5	0.9373	2.4128	4.2264
6	1.6385	2.1736	2.0697
7	0.1627	0.2510	2.6414
8	1.0931	1.3423	0.9836
9	0.9468	3.9102	1.7092
10	1.3080	1.5130	2.3380

The within-day and day-to-day precision for measurements of AP activity with tablets sealed in rotors was approximately the same as that found for SGOT. Precision for assay with the fresh tablets was low because of the high results for weeks 2 and 4 (see Table 2.14). Precision improved for both fresh tablets and Calbiochem kits when reagent blanks were subtracted; however, it decreased for in situ reconstitution of the tablets (see Table 2.15). Apparently, inter-tablet variability was the controlling factor in this instance. In summary, the stability study did indicate that the AP reagent tablets were stable for the 10-week period. Inter-tablet variability resulted in assays with slightly lower precision than those obtained with the commercial kits (see Table 2.16); however, results in general were quite satisfactory and demonstrated the overall feasibility for this assay. It was apparent that uniformly high-quality water will be required for tablet reconstitution to avoid excessively high reagent blanks.

Table 2.14. Day-to-day variation - alkaline phosphatase reagent tablet stability test

Std. No.	S.K. in situ			S.K. fresh			Calbiochem.		
	Mean	SD	RSD	Mean	SD	RSD	Mean	SD	RSD
1	16.80	1.44	8.59	22.71	5.91	26.00	14.59	1.09	7.46
2	40.33	2.89	7.16	46.95	5.97	12.71	31.45	1.60	5.08
3	77.19	5.70	7.39	81.95	6.26	7.63	55.53	1.62	2.91
4	104.78	6.48	6.18	110.36	6.48	5.87	75.58	6.07	8.04
5	142.89	8.05	5.64	147.73	11.59	7.85	104.71	3.05	2.91

Table 2.15. Day-to-day variation - alkaline phosphatase reagent tablet stability test (blank subtracted)

Std. No.	S.K. in situ			S.K. fresh			Calbiochem.		
	Mean	SD	RSD	Mean	SD	RSD	Mean	SD	RSD
1	15.06	1.43	9.46	17.51	1.01	5.77	12.04	0.62	5.14
2	38.60	3.33	8.62	41.74	2.65	6.36	28.90	1.17	4.05
3	75.45	5.70	7.55	76.75	4.84	6.30	52.98	1.61	3.04
4	103.04	7.28	7.07	105.16	7.46	7.09	73.03	5.58	7.63
5	141.14	8.29	5.87	142.52	11.96	8.39	102.16	3.33	3.26

Table 2.16. Comparison of precision data with three alkaline phosphatase reagent preparations (% RSD)

Reagent	within-day	Average day-to-day	
		blanks not subtracted	blanks subtracted
SKI tablets (in situ) ^a	2.87	6.99	7.71
SKI tablets (fresh) ^b	1.10	12.01	6.78
Calbiochem ^c	0.84	5.28	4.62

^aSealed rotors; tablets reconstituted individually at time of use.

^bSealed 20-tablet vials; vials opened and entire contents reconstituted at time of use.

^cCommercial reagent kits.

Evaluation of LDH-L reagent tablets. The final assay to be evaluated in this development of rotors preloaded with reagent tablets is the kinetic assay of LDH in lactate media (LDH-L). Smith-Kline supplied the following specifications and performance data for lot No. 30-4-70:

Specification

Measured

1. pH 8.8 ± 0.1

8.81 ± 0.01

n = 14
CV = 0.1%

<u>Specification (cont'd)</u>	<u>Measured (cont'd)</u>	
2. Weight: 10.0 ± 0.5 mg	9.97 ± 0.15 mg	n = 20 CV = 1.5%
3. Dissolution time	19.7 ± 4.9 sec	
4. Activity:		
a. Eskalab Abnormal Control No. 4448 30°C range = 172 to 242 IU	a. Serum/sample ratio = 1:35 (Our standard CFA product) Run on CFA 199 IU ± 6.6	n = 6 CV = 3.3%
	Serum/sample ratio 1:120 Run on Gilford Spectrophotometer 194 IU ± 12.3	n = 6 CV = 6.3%
	Serum/sample ratio = 1:11 Run on CFA With control diluted: 1:2 191.5 IU ± 2.0	n = 8 CV = 1.0%
	Serum/sample ratio = 1:12 Run on Gilford Spectrophotometer With control diluted: 1:2 168.6 IU ± 12.6	n = 6 CV = 7.5%
b. Eskalab Normal Control No. 4447 30°C range = 68 to 96 IU	b. Serum/sample ratio = 1:11 Run on CFA 76 ± 1.6 IU	n = 4 CV = 2.1%
	Serum/sample ratio = 1:12 Run on Gilford Spectrophotometer 67.8 ± 2.1 IU	n = 6 CV = 3.1%

The 10-week stability study on reagent tablets for LDH-L proceeded quite smoothly with none of the leaking-rotor problems previously encountered during some of the other stability studies.

The precision of within-day measurements (Table 2.17) was acceptable. Day-to-day variations displayed the expected higher variability for in situ reconstitution, probably due in large part to inter-tablet differences (see Table 2.18); however, the deviations for standards 3, 4, and 5 were only slightly higher than those observed for the fresh tablet and the Calbiochem reagents (see Table 2.19).

Although reagent formulation was exactly the same as that used in the Calbiochem reagent, assays averaged 21 to 26% lower activities with the tablets. The manufacturer also observed this difference but was unable to provide an explanation.

Mean within-day and day-to-day precision was approximately the same for the LDH-L assay, as was the precision observed for SGOT and AP (compare Tables 2.9, 2.16, and 2.20). The data indicated that reagent tablets for the assay of LDH-L were stable over a 10-week period when sealed in the cuvetts of CFA rotors. The assay performed with these tablets compared well with assays using fresh tablets. The reduction in measured activity for these tablets compared well with assays using fresh tablets. The reduction in measured activity for these tablets compared with those of the Calbiochem reagent kits having the same reagent formulation is puzzling; however, it is not a problem provided the assay is reproducible week after week, as demonstrated in these results.

2.3.2 Creatine phosphokinase isoenzyme measurement

Traditionally, the separation and measurement of isoenzymes have been performed by electrophoresis and densitometry. Recently, a method for the measurement of two isoenzymes of creatine phosphokinase (CPK) was proposed which appeared amenable to measurement on the CFA. This section describes studies performed to evaluate this methodology.

Table 2.17. Within-day variation - LDH-L reagent tablet stability test

Exp. No.	S.K. in situ			S.K. fresh			Calbiochem		
	Mean	SD	RSD	Mean	SD	RSD	Mean	SD	RSD
STANDARD No. 1									
1	24.4	1.0	4.1	24.8	0.2	0.7	34.8	0.5	1.5
2	20.9	1.8	8.5	26.9	1.2	4.3	36.7	0.5	1.3
3	20.6	3.0	14.3	24.3	0.2	0.8	32.0	0.4	1.2
4	21.6	1.1	5.0	24.9	0.6	2.2	34.6	0.3	0.8
5	23.7	1.0	4.2	26.5	1.0	3.7	32.8	0.3	0.8
6	24.5	1.2	4.9	22.6	0.3	1.1	32.8	0.2	0.5
7	20.5	0.7	3.5	24.2	0.2	1.0	33.1	0.2	0.6
8	22.6	1.4	6.3	22.6	0.2	0.7	30.3	0.2	0.6
9	20.0	2.0	10.0	23.2	0.7	2.9	30.3	0.3	1.0
10	24.6	2.0	8.2	22.4	0.1	0.4	30.0	0.2	0.5
STANDARD No. 2									
1	48.9	1.5	3.0	50.4	0.8	1.5	66.3	0.8	1.3
2	39.1	3.7	9.4	55.2	0.8	1.4	69.7	1.2	1.7
3	43.8	3.1	7.0	49.4	0.2	0.4	62.6	1.0	1.6
4	47.2	0.5	1.1	51.3	0.5	1.0	65.3	0.0	0.1
5	48.4	0.8	1.5	52.3	0.7	1.3	64.0	0.6	0.9
6	48.0	3.7	7.8	47.7	0.8	1.6	63.7	0.7	1.1
7	44.8	1.0	2.1	49.0	1.1	2.2	64.3	0.5	0.7
8	48.2	0.7	1.5	47.8	1.0	2.0	60.9	0.3	0.5
9	44.9	3.0	6.7	47.5	1.0	2.2	59.1	0.2	0.3
10	52.1	3.0	5.7	47.2	0.3	0.5	59.7	0.2	0.4
STANDARD No. 3									
1	73.4	2.5	3.4	72.7	0.3	0.5	92.4	0.7	0.7
2	67.0	0.8	1.3	77.6	1.8	2.3	98.2	1.7	1.7
3	66.4	3.0	4.4	71.8	1.1	1.6	90.3	0.4	0.5
4	70.2	1.2	1.7	75.7	0.1	0.2	95.2	0.2	0.2
5	71.8	2.9	4.1	74.3	0.7	0.9	90.4	0.3	0.3
6	70.3	1.6	2.2	68.9	0.5	0.7	88.9	0.4	0.4
7	61.8	1.9	3.1	71.2	0.5	0.8	93.1	0.7	0.7
8	66.1	2.3	3.5	67.8	0.6	0.9	86.5	0.2	0.2
9	68.7	2.4	3.4	68.5	0.6	0.8	85.3	0.4	0.5
10	82.1	0.8	1.1	68.8	0.7	1.1	85.3	0.7	0.8
STANDARD No. 4									
1	91.1	2.1	2.3	90.6	0.6	0.7	115.8	0.5	0.5
2	89.0	4.2	4.7	97.7	1.7	1.7	124.0	0.7	0.6
3	84.9	6.7	7.9	89.6	0.2	0.2	114.8	0.3	0.2
4	92.3	1.4	1.5	95.8	0.2	0.2	120.5	0.8	0.6
5	89.5	3.4	3.8	91.1	2.0	2.2	111.8	1.1	1.0

Table 2.17. (Continued)

Exp. No.	S.K. in situ			S.K. fresh			Calbiochem		
	Mean	SD	RSD	Mean	SD	RSD	Mean	SD	RSD
STANDARD No. 4 (continued)									
6	89.2	2.8	3.1	89.5	0.7	0.8	114.0	0.4	0.3
7	81.4	2.8	3.4	92.5	0.6	0.6	119.0	0.4	0.3
8	83.7	1.5	1.8	87.0	0.3	0.3	109.7	0.5	0.4
9	82.0	3.1	3.8	87.6	0.3	0.3	106.0	2.1	1.9
10	91.9	2.1	2.3	87.7	0.3	0.3	108.2	0.2	0.2
STANDARD No. 5									
1	135.1	2.4	1.8	133.8	0.4	0.3	170.7	0.9	0.5
2	127.1	2.8	2.2	142.0	2.8	2.0	180.5	2.2	1.2
3	117.1	1.5	1.3	124.6	0.9	0.7	161.9	1.0	0.6
4	122.1	3.4	2.8	132.7	0.3	0.3	169.0	0.6	0.3
5	127.3	0.6	0.5	131.2	1.7	1.3	158.4	0.5	0.3
6	124.0	2.4	2.0	121.6	0.9	0.7	156.8	0.2	0.1
7	108.5	4.8	4.5	127.2	0.2	0.1	161.4	0.7	0.5
8	120.0	2.7	2.3	123.1	0.2	0.2	155.0	0.9	0.6
9	118.1	2.7	2.3	122.9	0.1	0.1	149.6	0.8	0.5
10	130.1	2.7	2.1	122.8	0.4	0.3	149.5	0.5	0.4*

*

Table 2.18. Day-to-day variation - LDH-L reagent tablet blank absorbances

Exp. No.	S.K. in situ	S.K. fresh	Calbiochem
1	0.1273	0.1020	0.0892
2	0.1431	0.1011	0.0899
3	0.1443	0.0989	0.0904
4	0.1229	0.0997	0.0963
5	0.1400	0.0954	0.0837
6	0.1275	0.1018	0.0771
7	0.1055	0.0907	0.0854
8	0.1084	0.1012	0.0946
9	0.1649	0.0949	0.0931
10	0.1470	0.1076	0.0909
MEAN	0.1331	0.0993	0.0891
SD	0.0183	0.0047	0.0057
RSD, %	13.8	4.7	6.4

Table 2.19. Day-to-day variation - LDH-L reagent tablet stability test

Std. No.	S.K. in situ			S.K. fresh			Calbiochem		
	Mean	SD	RSD	Mean	SD	RSD	Mean	SD	RSD
1	22.33	2.24	10.03	24.21	1.62	6.69	32.74	2.14	6.53
2	46.52	3.98	8.55	49.77	2.56	5.14	63.55	3.13	4.93
3	68.79	3.80	5.52	71.74	3.30	4.60	90.55	4.16	4.60
4	87.47	4.85	5.55	90.90	3.50	3.85	114.36	5.53	4.83
5	122.93	7.64	6.21	128.17	6.44	5.03	161.29	9.48	5.88

Table 2.20. Comparison of precision data with three LDH-L reagent preparations

Reagent	Average within-day (% RSD)	Average day-to-day (% RSD)
SKI tablets (in situ) ^a	3.99	7.17
SKI tablets (fresh) ^b	1.10	5.06
Calbiochem ^c	0.69	5.35

^aSealed in rotors; tablets reconstituted individually at time of use.

^bSealed 20-tablet vials; vials opened and entire contents reconstituted at time of use.

^cCommercial reagent kits.

Background. Creatine kinase (creatine phosphokinase) has been demonstrated to occur in three major dimeric isoenzyme forms: CPK/MM (predominant in skeletal muscle), CPK/MB (found in myocardium), and CPK/BB (present in brain, lung, and smooth muscle tissue).^{7,8} The detection in serum of elevated levels of CPK/MB activity has received considerable attention as a specific and sensitive test of myocardial infarction.⁷⁻¹⁰

Several techniques have been employed to separate or selectively determine CPK/MB, present in normal sera only in trace amounts, from the much more abundant CPK/MM isoenzyme. Electrophoretic separation is generally considered to be the most definitive technique for confirming the occurrence of CPK/MB in serum;¹¹⁻¹³ however, the method requires special equipment and is relatively time-consuming. Results obtained by fluorometric scanning of the developed chromatogram are frequently only semiquantitative,¹⁴ and detection of CPK/MB requires that greater than ~ 12 IU/liter be present in the serum sample.¹⁵

Separation of CPK isoenzymes by ion-exchange column chromatography appears promising.^{11,12,15-17} Analysis is more rapid than in electrophoresis, and direct quantitation of CPK activity in column eluates is possible. However, poor separation of CPK isoenzymes with commercially produced column-chromatographic kits, caused by cross-contamination of isoenzymes within column eluate fractions, has been reported.¹⁸ Carry-over of CPK/MM into the CPK/MB fraction may occur, especially if the ratio of MM to MB is high,¹⁶ and carry-over of CPK/MB into the CPK/BB fraction is pronounced when CPK/MB activity is elevated.^{14,18} This may result in a false-positive diagnosis of myocardial infarction.^{14,19}

Recently, Rao et al.²⁰ introduced a diagnostic method based on the differential activation of CPK/MB by dithiothreitol (DTT). Two aliquots

of serum are obtained; one is treated with DTT and the other is not. Both samples are analyzed with a reagent containing a glutathione activator. The latter activator is sufficient to activate CPK/MM, whereas activation of CPK/MB in the presence of CPK/MM requires DTT. The activity of CPK/MB (A_{MB}) is estimated by the difference in activity of the treated sample (A_T) and untreated sample (A_U):

$$A_{MB} = A_T - A_U \quad (57)$$

The method has the attractive features of being simple and rapid to perform and, thus, well suited to stat applications. Again, however, the commercial adaptation of this procedure has received considerable criticism.²¹⁻²⁴

The primary objection expressed is that DTT activation is not particularly selective for CPK/MB but rather that all isoenzymes are further activated (relative to glutathione activation).^{17,22} Such a lack of selectivity would result in an overestimation of CPK/MB activity, and, thus, false-positive diagnosis of myocardial infarction is again possible.

We have evaluated the chemical activation procedure of Rao et al.²⁰ for measurement of CPK/MB activity by use of a miniature CFA system interfaced with a minicomputer (PDP 8/e, Digital Equipment Corporation, Maynard, Massachusetts).^{25,26} The advantages of the CFA for this procedure include small volume requirements (3 λ of sample and 30 λ of reagent are required per assay) and the ability to run replicates of sample, both treated and untreated with DTT activator, in parallel under identical conditions of temperature, etc., for high precision of rate measurement (generally better than 1% CV). We have considered the theoretical implications of rate-measurement precision on the reliability of estimates of CPK/MB by the method of Rao. In addition, we have developed a linear-

search computer subroutine to automatically locate the linear maximum rate segment of the kinetic data, with minimal error due to the different lag phases for treated vs untreated sample,²⁷ and to automatically compute the activity (and percent activity) ascribed to CPK/MB.

Analytical methods. Serum samples tested were lyophilized control serum (Statzyme) or serum from Ft. Sanders Presbyterian Hospital. The latter samples were referred to the hospital for determination of CPK isoenzymes by electrophoresis. Aliquots of these samples were frozen, without the addition of sulfhydryl reagent, for subsequent analysis via Rao's method and column chromatography.

Creatine phosphokinase activity was determined by the use of Calbiochem CPK/MB Stat Pack reagents. The reagent contains glutathione as the sulfhydryl activator. Samples were further activated by DTT reagent for determination of the total CPK activity.

Electrophoretic data were obtained on authentic patient samples at Ft. Sanders Presbyterian Hospital, Knoxville, Tennessee. Electrophoresis was performed on agarose gel using a Corning ACI system. The electrophoretograms were developed and scanned fluorometrically to obtain estimates of the relative activities of the CPK isoenzymes. Total CPK was estimated by a manual two-point kinetic procedure, and CPK/MB was estimated by the product of CPK/MB relative activity and the total activity of the sample.

The procedure employed for chemical activation (Rao's method) is in agreement with the recommendations of the reagent manufacturer (Calbiochem CPK/MB Stat-Pack) and of Rao et al.²⁰ Using the ORNL Sample-Reagent Loader,²⁶ the reagent is first concentrated by the addition of 3.1 ml of distilled water rather than the 15.5 ml recommended

by the manufacturer when following the manual procedure. The 17-place multicuvet rotor is loaded by charging 3 λ of sample (+50 λ diluent) and 30 λ of reagent, prepared as described above (+50 λ diluent) in the appropriate chambers.²⁶ The rotor and contents are placed on the analyzer and brought to $30.0 \pm 0.2^\circ\text{C}$ via radiant heating. Sample and reagent are transferred and mixed by a procedure of (1) rapid acceleration to 4000 rpm, (2) rapid deceleration to rest, and (3) adjustment of rotor speed to 1000 rpm. After a 30-sec delay, 16 absorbance measurements are taken at 30-sec intervals, each measurement being the average of 30 consecutive revolutions of the appropriate cuvet past the stationary phototube.

Data are automatically processed by a linear search computer subroutine (see Automated Data Analysis Sect.) and a program to compute CPK total activity, CPK/MB activity, and percent CPK/MB. The latter program requires that the rotor be loaded in the sequence; that is, aliquots of sample treated with DTT (treated sample) are loaded in duplicate, followed by aliquots of untreated sample. Dithiothreitol is added to an aliquot of sample at ice-bath temperature²⁷ in the proportions recommended by the reagent manufacturer.

Worthington CPK isoenzymes columns were also used. Eluent buffer solutions were adjusted in NaCl concentration according to the recent recommendations of the manufacturer^{28,29} for minimizing intermixing of CPK isoenzymes in column eluates. The treated sample (0.25 ml), prepared as in the dual activation procedure, was eluted by the discrete addition of 5.0 ml of pH 7.5 Tris buffer containing 0.3 M NaCl (MM buffer), followed by 3.0 ml of buffer containing 0.20 M NaCl (MB buffer), and finally 3.0 ml of buffer containing 0.3 M NaCl (BB buffer). Each fraction was collected in a tube, inverted several times to obtain homogeneity, and

activated by DTT reagent (as used in dual activation procedure) in the proportion of 10 λ DTT reagent per milliliter of eluate. Column eluate (45 λ plus 40 λ diluent) and reagent (30 λ plus 25 λ diluent) were loaded in a multicuvet rotor by the ORNL Sample-Reagent Loader. Data acquisition was identical to that described under dual activation procedure.

Automated data analysis. Figure 2.34 illustrates the reaction progress curves for the determination of CPK/MB by the dual activation procedure (Rao's method). Treated sample has been activated by glutathione and DTT, whereas the "untreated sample" has been activated by glutathione only. One immediately notices the difference in reaction lag phase, depending on the extent of activation of the sample. In addition, lag phase in CPK analysis can vary considerably from sample to sample.³⁰ Most automated enzyme analyzers, based on a simple two-point fixed-time analysis, will severely underestimate enzymatic activity when lag or depletion phases occur during the time that the reaction is being monitored. For this reason, Rao and Mueller have recommended that the chemical activation method not be used with such instrumentation.²⁷

The effect of reaction lag or a reagent depletion phase is corrected by visually selecting the appropriate segment of the progress curve for measurement of the rate of reaction, or by establishing criteria that will permit a laboratory computer to automatically perform this function.^{31,32} In our procedure for CPK determination, 16 absorbance data points are computed at precisely timed intervals for each of the 17 cuvetts of the multicuvet rotor disc, and the data are stored by the computer. A linear least-squares regression analysis subroutine is used to compute a slope (enzyme rate) and correlation coefficient for the first eight data intervals. The correlation coefficient computed is compared to a "minimum

Fig. 2.34. Reaction progress curves for creatine phosphokinase via the chemical activation procedure (Rao's method). Untreated sample - activation by glutathione only; treated sample - activation by dithiothreitol and glutathione. Arrows on the curves enclose the segment of the progress curve selected by the computer subroutine for reaction rate calculation.

acceptable correlation coefficient" selected by the operator. If the computed coefficient is less than this criterion, the slope is set equal to zero; otherwise, the computed value is retained. The interval used, slope, and correlation coefficient are stored in computer memory. The process is repeated for intervals 2-9, 3-10, ... 9-16. The list of computed enzyme rates are searched for the maximum rate, and the results are presented on a teletype. If none of the intervals had a correlation coefficient that exceeded the criterion selected by the operator, the teletype flags the result by typing "DATA DOES NOT MEET LINEARITY CRITERION."

The format of the program is illustrated in Table 2.21 for eight serum samples. The rotor is loaded with duplicates of each treated sample followed by duplicates of untreated sample. An additional portion of the computer program averages the activities of the replicates and computes CPK/MB as the difference in activity of the treated and untreated sample. In Fig. 2.34, the arrows enclose the segment of reaction rate data selected by the computer for use in computation of the enzymatic rate. It is seen that neither lag nor depletion phases were included in the data selected for this computation. Analysis of CPK/MB is rapid by the CFA-computer system; actual data taking requires 8-1/2 min, and the computed results of all 16 analytical cuvetts are obtained in an additional 10 min.

Statistical prediction of analytical error. As indicated in Eq. (57), the activity of CPK/MB (A_{MB}) is computed from the measurement of the activity of treated (A_T) and untreated (A_U) sample. The significance of the computed result is dependent on the precision of the measurements of A_T and A_U . This precision may be expressed as the standard deviation(s) of the estimate, for example,

Table 2.21. Typical output of chemical activation procedure on a Centrifugal Fast Analyzer system

NUMBER OF CUVETS UNDER CONSIDERATION: 17
 NUMBER OF SETS OF OBSERVATIONS: 16
 DELAY INTERVAL: 180
 OBSERVATION INTERVAL: 30
 RUN NUMBER: 1
 GEMSAEC UNIT: 17.1
 WHICH CUVET CONTAINS BLANK: 1
 MINIMUM ACCEPTABLE CORRELATION COEFFICIENT: 0.995
 ENZYME FACTOR: 15330

Cuvet	Time interval (sec)	Enzyme rate (ODU/min)	Activity (M mole/min)	Correlation coefficient
2	180 - 420	0.046262	709.19800	1.0000
3	180 - 420	0.046372	710.88300	1.0000
4	300 - 540	0.043927	673.39400	1.0000
5	330 - 570	0.043808	671.57900	1.0000
6	390 - 630	0.004795	73.49900	0.9988
7	300 - 540	0.004925	75.49360	0.9989
8	300 - 540	0.004403	67.49000	0.9996
9	390 - 630	0.004457	68.32160	0.9994
10	180 - 420	0.080230	1229.92000	0.999
11	180 - 420	0.081436	1248.41000	0.9999
12	240 - 430	0.072698	1114.46000	1.0000
13	240 - 480	0.072777	1115.67000	1.0000
14	210 - 450	0.003800	58.25210	0.9988
15	210 - 450	0.003786	58.03670	0.9981
16	300 - 540	0.003291	50.45630	0.9981
17	330 - 570	0.003356	51.44100	0.9985 ^a

^aL G 18,G

Determination of CPK isoenzymes by differential activation

NUMBER OF DIFFERENT SAMPLES: 4
 NUMBER OF REPLICATES EA., TREATED AND UNTREATED: 2
 NUMBER OF CUVETS UNDER CONSIDERATION: 17

Sample number	Activity, IU/l CPK, total	Activity, IU/l CPK/MB	% Activity CPK/MB
1	710.04	37.56	5.29 -- high value
2	74.50	6.59	8.85 -- high value
3	1239.17	124.10	10.02 -- high value
4	58.15	7.20	12.38 -- high value ^a

^aAll samples are loaded in duplicate, treated samples followed by duplicate, untreated samples.

$$S = \left[\sum_{i=1, n} \frac{(X_i - \bar{X})^2}{n-1} \right]^{1/2}, \quad (58)$$

where X_i is an individual measurement, $\bar{X}$ is the arithmetic mean, and n is the number of replicate measurements. The precision of the activity measurements of treated and untreated sample may also be expressed in a relative manner by the coefficient of variation:

$$CV_T = \frac{S_T \cdot 100}{A_T} \quad (59)$$

$$CV_U = \frac{S_U \cdot 100}{A_U}. \quad (60)$$

From the theory of propagation of random errors in computations,^{33,34} the error in determining A_{MB} from Eq. (57) may be estimated from the equation

$$S_{MB}^2 = S_T^2 + S_U^2, \quad (61)$$

where S_{MB}^2 , S_T^2 , and S_U^2 are estimates of the variances of A_{MB} , A_T , and A_U , respectively. Combining Eqs. (59) through (61), one obtains:

$$S_{MB} = \frac{1}{100} \sqrt{A_T^2 CV_T^2 + A_U^2 CV_U^2}. \quad (62)$$

If A_T and A_U are comparable in magnitude, the relative error in their measurement may be approximated:

$$CV_T \approx CV_U = CV_{meas}. \quad (63)$$

Expressing the error in A_{MB} in terms of a coefficient of variation (CV_{MB}) and using the approximation of Eq. (63), Eq. (62) becomes:

$$CV_{MB} = \frac{CV_{meas}}{A_{MB}} \sqrt{A_T^2 + A_U^2}, \quad (64)$$

or, in terms of the fraction, X , of CPK/MB relative to total CPK:

$$CV_{MB} = \frac{CV_{meas}}{X} \sqrt{1 + (1 - X)^2}. \quad (65)$$

Some consequences of Eq. (65) are illustrated in Fig. 2.35. The reliability of estimates of CPK/MB by the chemical activation procedure is a sensitive function of the precision of the measurement of the rates of treated and untreated samples. For the manual performance of this assay, the average CV for rate measurement is 4.9%.³⁵ From Fig. 2.35, one thus predicts that manual procedure estimates of CPK/MB are highly unreliable ($CV_{MB} > 100\%$) for CPK/MB activities less than $\sim 7\%$ of total CPK.

Table 2.22 lists the average CV_{meas} for the chemical activation protocol using the miniature CFA (including automatic loading of sample and reagent, rate selection by linear search computer subroutine, etc.). For total CPK activities in the elevated range (>80 IU/liter), the system precision is generally better than 1%.

Analytical results by the dual activation procedure. Table 2.23 illustrates the correspondence of CPK/MB estimates in fresh sera via electrophoresis with the chemical activation procedure using thawed aliquots of sera. Reference-data total CPK values were determined by a manual procedure and may represent serious underestimation of total activity for >400 IU/liter due to substrate depletion (e.g., samples 8 and 9). Reference estimates of CPK/MB activity are made from the relative peak areas of the electrophoretogram and thus may be considered

Fig. 2.35. Estimated precision for the determination of CPK/MB as a function of precision of rate measurement and relative activity of CPK/MB.

Table 2.22. Precision of linear search program and centrifugal fast analyzer system for the determination of creatine phosphokinase enzymatic rate

Enzymatic rate (ODU/min)	Corresponding CPK activity range ^a (IU/liter)	Average coefficient of variation (%)
0.001-0.005	14-71	2.15
0.005-0.010	71-142	1.24
0.010-0.050	142-712	0.69
0.050-0.100	712-1420	0.81

^aCPK activity, IU/liter = ODU/min x enzyme factor;
for dual activation protocol, enzyme factor = 14255.

Table 2.23. Comparison of electrophoresis and Rao method for the determination of CPK-MB isoenzyme

Patient No.	Reference Method		Centrifugal Fast Analyzer	
	CPK total ^a	CPK MB ^b	CPK total ^c	CPK MB ^d
1	238	28	290.9 ± 1.6 ^e	20 ± 1.8 ^e
2	405	22	400.9 ± 0.1 ^e	33.3 ± 1.7 ^e
3	52	0	45.0 ± 0.4 ^e	4.4 ± 4.8 ^e
4	70	0	74.5 ± 1.4 ^e	6.6 ± 1.5 ^e
5	55	0	58.0 ± 0.2 ^e	7.1 ± 0.7 ^e
6	25	0	21.8 ± 1.0 ^e 23.8 ± 0.6 ^f	5.2 ± 3.5 ^e 3.5 ± 1.3 ^f
7	205	0	205.5 ± 1.0 ^f	20.3 ± 1.2 ^f
8	434	56	710.0 ± 1.2 ^e 708.6 ^{f,g}	37.6 ± 1.8 ^e 42.5 ^{f,g}
9	660	239	1239.2 ± 13.1 ^e 1438.9 ^{f,g}	124.1 ± 13.1 ^e 189.9 ^{f,g}
10	1000	0	942 ^{f,g}	151.3 ^{f,g}

^aManual kinetic method using Calbiochem reagent kit with both glutathione and DTT activation.

^bRelative peak areas of MM and MB isoenzymes determined from electrophoretogram (agarose gel) via fluorescence densitometry.

^cAutomated kinetic method using reagents in footnote a.

^dDetermined by difference in activity between aliquots activated by glutathione only and by glutathione plus DTT. Standard deviation of MB estimated by propagation of error.

^eSample volume = 2 µl.

^fSample volume = 5 µl.

^gSample diluted 1:5 with 0.9% saline prior to analysis.

only semiquantitative.¹⁴ For samples 3-6, no CPK/MB peak was detected by electrophoresis. The corresponding activity values by Rao's method ranged from 3.5 to 7.1 IU/liter CPK/MB (8 to 24% of total CPK), which were too low for detection by electrophoresis.¹⁵ However, no electrophoretically detectable CPK/MB was reported for sample 10, whereas the Rao method estimated a highly significant 151 IU/liter, or 16% of total. This magnitude of CPK/MB activity should easily have been detected by the electrophoretic procedure, and, thus, the results of the chemical activation procedure would be interpreted as false-positive for this sample.

Table 2.24 compares the results of the chemical activation procedure for determination of total CPK and CPK/MB with values obtained by ion exchange chromatography using a commercial kit procedure (Worthington CPK isoenzymes columns) and a control serum sample. The column eluates obtained by discrete chromatography using modified elution buffers (as described in Sect. 2.3.2)^{23,29} were treated as in the chemical activation procedure for the original sample. Two features of interest are noted. First, by our chromatographic procedure, CPK/MM had not been completely eluted within the fraction collected for its determination, as noted by other researchers using a similar technique.¹⁸ The differential activation of this eluate fraction by addition of DTT is approximately 2%, somewhat less than the 5% differential activation reported by Morin¹⁷ and much less than the 13 to 20% differential activation reported by Sheehan and Leipper²² for purified CPK/MM isoenzyme. It should also be noted that all estimates of CPK/MB isoenzyme activity and total CPK activity, including both treated and untreated column eluates, are within the extremely broad range of acceptable assay variation established by the control serum manufacturer.

Table 2.24. Determination of creatine phosphokinase isoenzyme in control serum

	Manufacturer's stated range ^b	Column Chromatography ^a		Percent activation ^e	Chemical activation
		Treated eluate ^c	Untreated eluate ^d		
CPK/MM	112 ± 6	86.0 ± 1.3	84.3 ± 0.7	2.0	110.7 ± 1.2 ^{d,f}
IU/liter	(96-123) ^g				
CPK/MB	9.8 ± 1.7	15.6 ± 0.1	8.4 ± 1.5	86.2	8.6 ± 1.8 ^f
IU/liter	(4.7-17) ^f				
CPK/BB	3.4 ± 1.6	8.2 ± 0.4	6.1 ± 1.3	33.9	
IU/liter	(1-8) ^g				
CPK, total	124 ± 10.4	109.8 ± 1.7 ^h	98.8 ± 2.1 ^h		119.3 ± 1.4 ^c
IU/liter	(90-145) ^g				

^a Ion exchange chromatography on Worthington CPK isoenzymes columns using modified buffer eluates.

^b Worthington Statzyme lot No. 76B112. Assay values established by ion exchange chromatography technique.

^c Activated by DTT and glutathione.

^d Activated by glutathione only.

^e Percent increase in activity after treatment of sample with DTT.

^f Activity of untreated sample attributed to CPK/MM. Difference in activity of treated and untreated sample attributed to CPK/MB (standard deviation estimated by propagation of error).

^g Manufacturer's stated range of acceptable assay variation.

^h Estimated by the sum of activity in column eluates (standard deviation estimated by propagation of error).

However, the activity attributed to CPK/MB by the chemical activation procedure appears somewhat low compared to the sum of anticipated results for CPK/MB and CPK/BB (both of which are substantially activated by DTT¹⁷).

The comparison of results for CPK/MB on patient sera as determined after ion exchange chromatography on Worthington CPK isoenzyme minicolumns with those obtained by the Rao method is presented in Table 2.25 for sera from patients with an electrophoretically confirmed CPK/MB activity. The total CPK activity reported for the column chromatographic technique is the sum of the total activity in the individual eluate fractions and, despite well-known dilution effects on CPK activity,¹⁷ is in good agreement with the direct estimate of total CPK activity. Considerable activity was found in the CPK/BB column eluate fraction (especially sample 4), despite the absence of electrophoretically detectable CPK/BB. It is inferred that the column eluate fractions collected are heterogeneous with respect to CPK isoenzymes, a phenomenon noted previously with commercial chromatographic kits.¹⁸

Summary. We have demonstrated that in order to obtain a reliable estimate of the difference in activity of treated and untreated samples in the chemical activation method of Rao, (1) the possible effect of lag and/or depletion phase during the interval that the reaction rate is being monitored must be considered, and (2) the precision of rate measurement must be high (preferably $\pm 1\%$ or better). If these criteria are not realized, then the magnitude of CPK activity attributed to CPK/MB [Eq. (57)] may be in serious error. It should be noted also that the activity of CPK/BB, if present, would contribute to the activity attributed to CPK/MB by the chemical activation procedure.¹⁷ However, this contribution is

Table 2.25. Comparison of column chromatographic and Rao method for the determination of CPK-MB isoenzyme in sera from patients with electrophoretically confirmed CPK-MB^a

Sample	Column Chromatography ^b			Rao Method	
	CPK, total (IU/liter)	CPK, MB (IU/liter)	CPK, BB (IU/liter)	CPK, total (IU/liter)	CPK, MB (IU/liter)
1	795.2	90.5 ± 2.3	56.2 ± 0.9	782.9	103.3 ± 13.5
2	1301.2	136.8 ± 2.0	49.5 ± 2.3	1165.3	170.0 ± 9.7
3	1162.2	85.9 ± 7.4	55.7 ± 2.5	1149.3	145.3 ± 9.3
4	1620.7	71.6 ± 0.5	70.7 ± 2.4	1446.0	449.4 ± 14.4

^aCPK-MB isoenzyme detected from fluorescence densitometry of electrophoretogram on agarose gel. No significant CPK-BB band was detected for any of the samples reported. Relative peak area of CPK/MB for these samples ranged from 28 to 32% of total.

^bDiscontinuous chromatography on Worthington CPK isoenzymes column. Creatine phosphokinase activity (total) is determined from sum of activities of MM, MB, and BB eluate fraction activated by dithiothreitol. CPK-MM is eluted with 5.0 ml of pH 7.5 Tris buffer containing 0.03 M NaCl, CPK-MB is eluted with 3.0 ml of buffer containing 0.20 M NaCl, and CPK-BB is eluted with 3.0 ml of buffer containing 0.3 M NaCl (see reference 29).

expected to be minor since CPK/BB is seldom detectable (i.e., <5 IU/liter) in serum, even in patients with diseases of tissues and organs containing a large proportion of this isoenzyme.³⁶

Although we have established conditions for the "reliable" estimation of the difference in CPK activity of a sample activated by glutathione and by glutathione plus DTT, the interpretation of this differential activity as CPK/MB activity is still uncertain. The relatively small number of samples examined in this report does not permit a conclusive statement concerning the utility of the chemical activation procedure for the determination of myocardial infarction. However, from the results reported here and elsewhere^{21,22} it appears that myocardial infarction cannot be reliably diagnosed solely on the basis of the magnitude of CPK/MB activity determined by the Rao method, at least not with the same criteria used in the reference techniques. For example, the normal range for CPK/MB via the Calbiochem adaptation of the Rao procedure is reported to be 0 to 15 IU/liter (total CPK $\leq$ 80 IU/liter),³⁵ whereas the corresponding range for well-controlled column chromatographic determinations is 0 to 2 IU/liter.^{15,16} The chemical activation procedure may be of value in confirming myocardial infarction by demonstrating a peaking of apparent CPK/MB activity 6 to 12 hr following the onset of chest pain.^{20,28}

2.3.3 Biochemical markers of cancer - measurement of gamma-glutamyl transpeptidase, leucine aminopeptidase, and 5'-nucleotidase

Recently there has been a great deal of interest in the search for biological markers that may be used as sensitive and noninvasive measures of malignant cell population. Thus, following surgical or chemotherapeutic treatment of a malignancy, such biochemical markers could be used to determine whether the treatment had successfully arrested tumor growth, as

indicated by their decreasing or stable concentrations. Several of the serum enzymes may be useful in this context. For example, serum levels of the exopeptidase enzymes gamma-glutamyl transpeptidase (GGTP, EC 2.3.2.2), leucine aminopeptidase (LAP, EC 3.4.11.1) and 5'-nucleotidase (5'-Nase, EC 3.1.3.5) are elevated above the upper limit of the normal range in carcinoma involving the hepatobiliary duct system.³⁷ No one marker is sufficient for monitoring a malignant cell population in all cases; therefore a battery of enzyme assays is recommended. Such a battery may include the exopeptidases mentioned above plus the less specific enzymes glutamate-oxaloacetate transaminase (GOT, or L-aspartate:2-oxoglutarate aminotransferase), AP, and LDH, all of which are of some utility in the diagnosis of cancer.³⁸ The latter three enzymes have previously been adapted to the CFA.^{3,39} A kinetic procedure for GGTP has also been adapted.^{2,3} In the present report, emphasis will be given to the description of the assay procedures for LAP and 5'-Nase.

GGTP. Results obtained using a commercially available reagent utilizing the substrate L-glutamyl-p-nitroaniline have been presented previously.³ This substrate was found to have limited solubility and stability. A new substrate, L-glutamyl-2-amino-5-nitrobenzoate, is now available in kit form from Bio-Dynamics/bmc. The normal range established using this reagent for nonfasting females was 0 to 33.3 IU/liter, nearly identical to the range of 8.2 to 38.3 IU/liter established with the original reagent.³ The distribution of results using the improved substrate is shown in Fig. 2.36 and summarized in Table 2.26

Fig. 2.36. Histogram showing frequency distribution of gamma-glutamyl transpeptidase activities for normal nonfasting female subjects.

Table 2.26. Serum gamma-glutamyl transpeptidase activities for apparently normal, nonfasting female human subjects

Group description	Number of subjects	GGTP activity (IU/liter at 30°C)		
		Mean	Median	Range
Females	36	12.5	8.1	1.9-42.0
20-29 years	16	8.9	8.9	1.9-16.7
30-39 years	5	8.1	7.4	2.0-15.2
40-49 years	4	10.9	7.8	6.4-17.2
50-59 years	8	15.9	13.1	4.7-40.3
> 60 years	3	30.9	41.5	9.2-40.0

LAP. A distinction must be drawn between "classical" leucine aminopeptidase, utilizing leucinamide as a specific substrate, and the group of amino acid arylamidase enzymes active toward synthetic substrates such as L-leucine-p-nitroanilide.^{37,40} It is the latter group of enzymes that is of diagnostic value in clinical chemistry; "true" LAP manifests only slight activity in human sera. We will follow clinical tradition and refer to the activity toward synthetic substrates as LAP activity, with the understanding that this activity is not due to a single enzyme and that true LAP is probably not involved.

So-called LAP can be detected with varying activity in nearly all human tissues, occurring principally in the mucosa of the small intestine and in the pancreas.^{37,41} Leucine aminopeptidase is elevated in disseminated malignant disease, including carcinoma of the head and neck, GI tract, bronchus, prostate, GU tract other than prostate, hematologic malignancies, and malignant melanoma.⁴² It may also be elevated in patients

with nonmalignant disease, especially of the liver.⁴² Leucine aminopeptidase activity also rises progressively as pregnancy advances, reaching several times the upper limit of the normal range (nonpregnant subjects) at the end of pregnancy.⁴³ This elevation is due primarily to a heat-labile placental LAP, which may be differentiated from normal serum LAP (heat-stable) by incubation at 60°C for 30 min.⁴³ Thus, by itself, elevated LAP activity in serum lacks specificity as a diagnostic test for the presence of tumor. In this respect, it has been indicated that elevated LAP in 24-hr urine specimens, as opposed to sera, may be of greater specificity in estimating the extent of the tumor and the success of treatment.⁴² The assay of serum LAP in connection with a battery of other exopeptidase enzyme assays greatly enhances its utility as a marker for cancer (see Table 2.27).

We have adapted the procedure of Szasz,⁴⁴ using the substrate L-leucyl-p-nitroanilide and monitoring the reaction at 400 nm:

The substrate concentration and its preparation (5.60 mM L-leucyl-p-nitroanilide in 0.466 M phosphate buffer, pH 7.2) in the buffer are as follows: Dissolve 0.0806 g L-leucyl-p-nitroanilide hydrochloride in ~ 0.5 ml of 95% ethanol. Add 10 ml of monobasic 0.466 M KH_2PO_4 (15.85 g/250 ml H_2O) and mix. Next add 25 ml of dibasic 0.466 M K_2HPO_4 (20.29 g/250 ml H_2O). Adjust to 50.0 ml with 0.466 M phosphate buffer (pH 7.20).

L-leucyl-p-nitroanilide (substrate for LAP) is much more soluble than L-glutamyl-p-nitroanilide (substrate for GGTP); however, it is sparingly soluble in neutral solution. The dissolution scheme above

Table 2.27. Properties of three biliary tract enzymes^a

	LAP	5'-Nase	GGTP
Technical facility of assay	Simple	Simple (uv)	Simple
Age dependency of serum activity	Significant until year puberty	Increased, but less significant until near puberty	Adult activity reached by 4 months
Pregnancy effect on serum activity	Significant	Not affected	Not affected
Specificity for hepatobiliary disease	High, but not exclusive	Nearly exclusive	Least specific of the group, yet high
Sensitivity for hepatic neoplasms	Greater than AP, especially in the anicteric patient	Same	Same
Existence of multiple molecular forms (molecular heterogeneity)	Yes	Yes	Yes

^aTaken from J. G. Batsakis, ref. 37.

allows the substrate to be dissolved first in acidic medium (high solubility) prior to neutralization with basic medium. Direct addition of a concentrated alkali, for example, KOH, can cause decomposition and loss of solubility of the substrate. Potassium phosphate buffer is preferred to the corresponding sodium ion buffer due to its superior solubility at the lower temperatures used for storage.

The buffered substrate prepared as described above was stable for ~ 2 weeks when stored in a refrigerator (4 to 6°C) between uses (see Fig. 2.37). The reaction blank absorbance with this substrate increased by 0.002 ODU/day (400 nm) when stored at 4 to 6°C and by 0.010 ODU/day when stored in the dark at 19°C. Szasz⁴⁴ found that his substrate (prepared in 0.05 M Tris, pH 7.2) increased in color by 10% per day when stored in the dark and the absorbance doubled within 2 hr when exposed to light. Although we also found that the blank absorbance did increase as the reagent ages, it did not affect the measured enzyme rates.

Ten microliters of sample (+40λ of diluent) are loaded into the multicuvet rotor. The initial reaction substrate concentration is 1.6 mM, in accordance with the procedure of Szasz.⁴⁴ After a 30-sec delay interval, the reaction is monitored at 400 nm at 30-sec intervals for a total of 16 observations. A linear search subroutine is used to select the data interval used in activity determination (enzyme factor = 2828).

A commercial kit for the analysis of LAP was purchased in order to determine its adaptability to the CFA. The kit consisted of lyophilized L-leucine-p-nitroanilide (and sodium phosphate) plus a reconstituting buffer, labeled-sodium phosphate, 72 mM, pH 7.2. However, the pH of the buffer was found to be 8.8, and only when the reagent was prepared

ORNL-DWG 76-14736R1

LAP REAGENT STABILITY
SAMPLE: PRECINORM
REAGENT: $S_0 \approx 1.6$ mm

"I-BARS" = 95%
CONFIDENCE INTERVAL,
INDIVIDUAL DAYS
RESULTS.

SHADED AREA = 95%
CONFIDENCE INTERVAL
OF DAY MEAN RESULTS.

Fig. 2.37. Effect of reagent storage conditions on the determination of serum leucine aminopeptidase activity. Separate aliquots of frozen control sample were thawed out for each day's analysis.

according to the directions for a macrotest did the resulting pH approach 7.2. Because of this, it is difficult to prepare a concentrated reagent (with the proper pH) for use with the automated sample-reagent loading system.

When performed according to the manufacturer's specifications, the initial reaction substrate concentration (S_0) is 0.8 mM. In Fig. 2.38, apparent LAP activity is given as a function of S_0 , using a "homemade" substrate solution. The sample is a control serum (Precipath) supplied by Boehringer Mannheim and assigned a LAP activity target value based on their kit procedure. It is obvious that: (1) experimental results are sufficiently within the manufacturer's stated acceptable assay variations at $S_0 = 0.8$ mM and (2) that 0.8 mM is a suboptimal substrate concentration. All subsequent LAP determinations reported are for homemade substrate solutions, giving reaction concentrations of 1.6 mM substrate and 0.1 M potassium phosphate buffer (pH 7.2).

Fifteen sera from normal, nonfasting female subjects (once-frozen) were tested. The distribution appeared very Gaussian, and the 95% range ($\bar{x} \pm 2\sigma$) was 15.0 to 28.6 IU/liter at 30°C. Szasz,⁴⁴ using an identical substrate and substrate concentration, found a range of 9.8 to 20.5 IU/liter at 25°C for female subjects. The package insert for the commercial kit tested reports a normal range (male and female) of 12 to 33 IU/liter at 30°C.

5'-Nase. 5'-Nucleotidase has a wide tissue distribution but is highest in liver, lung, brain, and kidney.³⁷ 5'-Nase has been frequently used as a biochemical marker for plasma membrane, since one form of the enzyme is largely restricted to the plasma membrane of liver cells and

Fig. 2.38. Effect of substrate concentration (L-leucine-p-nitroanilide) on the determination of serum leucine aminopeptidase activity. Results obtained were sufficiently within the manufacturer's target range at the specified substrate.

bile canaliculi.⁴⁵ The following reaction is catalyzed by 5'-Nase:

The clinical significance of elevated 5'-Nase activity is due to its high specificity for hepatobiliary disease.³⁷ 5'-Nase and LAP appear to be equally sensitive for the detection of hepatic metastases and exceed the sensitivity of nonspecific AP.³⁷ In contrast to LAP, 5'-Nase is unaffected in pregnancy.³⁷ Both AP and 5'-Nase are elevated in liver disease, while 5'-Nase is rarely elevated in bone disease.⁴⁵ Thus, an elevated serum AP level in the presence of a normal 5'-Nase level is usually an indication of bone disease, and where both are raised, hepatobiliary disease is virtually certain.⁴⁵ Serum 5'-Nase rather than AP should be estimated in children or pregnant women suspected of having liver disease.⁴⁶

Although determination of 5'-Nase is more specific than AP, AP is also capable of hydrolyzing the substrate for 5'-Nase (AMP). Thus, estimation of 5'-Nase activity on the basis of inorganic phosphate liberated [Eq. (67)] is greatly subject to error. Indeed, methodological difficulties in the estimation of true 5'-Nase activity have limited its acceptance as a diagnostic tool.

Equation (67) above may be linked with the following reaction:

where ADA is adenosine deaminase (EC 3.5.4.4). The ammonia liberated (NH₃) has been determined by the Berthelot reaction⁴⁶⁻⁴⁸ (requiring separate determination of the sample blank reaction) and kinetically by

use of L-glutamate dehydrogenase (GLDH):^{49,50}

In Eq. (68) above, the reaction is monitored by the decrease in absorbance at 340 nm due to the consumption of NADH. This latter procedure requires lengthy preincubation of the sample to eliminate interference due to endogenous NADH-consuming enzymes and substrates. In addition, preincubation eliminates the effect of endogenous NH_3 in the serum samples so that the measured rate is proportional to 5'-Nase activity. In the above procedures, an excess of a "diversionary" substrate for AP, usually phenylphosphate or beta-glycerophosphate, is added to the reaction medium to reduce the hydrolysis of the specific substrate (AMP) by AP.^{45,48}

A much simpler assay is based on the direct monitoring of the decrease in absorbance at 260 to 265 nm for the reaction in Eq. (68) due to the conversion of adenosine to inosine.⁵¹ When beta-glycerophosphate is added as an alternative substrate for AP, this assay is the most direct, selective procedure available for the determination of 5'-Nase, the chief limitation to its widespread use in clinical chemistry being the requirement of a suitable uv spectrophotometer.

In the multipurpose optical modification of the CFA,¹ light from an external source, such as a 200-W xenon-mercury source coupled with a small monochromator and a stabilized dc power supply, can be directed via a quartz fiber optic bundle to permit absorbance measurements in the uv spectral region. A rotor having quartz windows for transmission of uv radiation is used. The linearity of the optical system for adenosine is illustrated in Fig. 2.39, and photometric performance for standard solutions of adenosine and inosine is summarized in Table 2.28.

Fig. 2.39. Linearity of absorbance data for adenosine using multi-optical configuration of miniature Centrifugal Fast Analyzer (xenon-mercury source and quartz annulus rotor).

Table 2.28. Optical performance of the Centrifugal Fast Analyzer in ultraviolet spectral range -- determination of molar absorptivity for adenosine and inosine

Wavelength	Adenosine	Inosine	Adenosine-Inosine
260 nm (CFA)	15.5×10^3	6.62×10^3 ^a 6.66×10^3 ^b	8.81×10^3
260 nm ^c	15.4×10^3		
265 nm (CFA)	14.1×10^3	5.98×10^3	8.12×10^3
265 nm ^d			8.10×10^3

^aEstimated from absorbance of stock solutions of inosine.

^bEstimated from absorbance of stock solutions of adenosine in the presence of adenosine deaminase.

^cLiterature data included with commercial adenosine deaminase (Prod. No. A-0387), Sigma Chemical Co., St. Louis, Mo.

^dLiterature obtained from H.-U. Bergmeyer (ed.), pp. 1004-6, Methods of Enzymatic Analysis, Academic, New York, 1965.

The reagent preparation is as follows [buffer: 0.233 M Tris (28.39/liter) plus 0.0467 M $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (11.5 g/liter), pH 7.9]: stock AMP solution (4.66 mM:0.162 g AMP) is prepared in 100 ml buffer. Stock beta glycerophosphate (78.2 mM:0.845 g) is prepared in 50 ml buffer. The combined reagent, which includes adenosine deaminase (Type III), contains 0.27 ml stock AMP plus 2.25 ml stock beta-glycerophosphate plus 25 λ adenosine deaminase. Glycerophosphate stock is stable for months, AMP stock is stable for several weeks, adenosine deaminase in glycerol suspension is stable for at least 6 months to 1 year, and combined reagent is stable 1 to 2 weeks. All solutions are stored at 4 to 6°C.

Unlike LAP and GGTP, none of the commercially available serum control samples tested were found to have a measureable 5'-Nase activity. As a

result, a stock solution of adenosine, $4.67 \times 10^{-3} \text{ M}$ (0.1247 g/100 ml buffer), is used to check the viability of ADA in the combined reagent. When the sample is used in the protocol described below, a reaction absorbance of ~ 0.4 ODU is obtained with active ADA, and ~ 0.9 ODU is obtained in the absence of ADA activity.

Four microliters of serum (+ 50 λ of diluent) and 30 λ of combined reagent (+ 50 λ of diluent) are loaded into the multicuvet rotor. Although a decrease in absorbance is measured, a reagent blank may be used due to the high absorbance contribution of serum protein at 260 nm. Samples should be treated by high-speed centrifugation prior to analysis to remove interference by particulates or gross lipemia which would scatter light at this wavelength. A broad band-pass filter (200 to 400 nm) is used as a secondary filter to isolate the optics from stray room lighting, and a narrow slit width (0.5 nm) is used at the source. Data are obtained at 1-min intervals over the interval 5 to 15 min (enzyme factor = 8843), each reading being the average of 30 consecutive revolutions of the rotor (rotor speed 1000 rpm) past the stationary photomultiplier.

Due to the relatively noisy xenon-mercury source, a minimum rate of 0.001 ODU/min (± 8 IU/liter) was judged to be the minimum measurable enzyme activity. Nineteen sera samples from apparently healthy, normal nonfasting female subjects were tested; 63% of the sera had ≤ 8 IU/liter. Only one sample (5%) had an activity exceeding 20 IU/liter, which is taken as the upper limit of the normal range. Belfield and Goldberg,⁵¹ using a similar procedure, found a range of 0 to 25 IU/liter in female subjects, with only 4% having an activity exceeding 15 IU/liter. Precision in the measurable normal range, 8 to 20 IU/liter, is modest, averaging ± 1.5 IU/liter (± 0.0002 ODU/min).

Preliminary screening of serum samples. Table 2.29 summarizes results from a preliminary examination of samples provided by the National Cancer Institute, Bethesda, Maryland. Sample 125 is identified as a serum from a patient with known liver carcinoma. For this sample, elevated activities of GGTP, LAP, and 5'-Nase were noted. The remainder of the samples listed were not identified. Gamma-glutamyl transpeptidase and LAP activities were elevated in eight and ten cases, respectively, of the 22 samples tested and, in general, parallel one another; 5'-Nase activity was elevated in only two cases. This may be a reflection of the greater specificity of the latter enzyme, or a loss of activity in sample handling and storage. Other researchers have found that elevation of LAP activity is paralleled by rises in 5'-Nase activity in diseases of the pancreas and hepatobiliary tree.⁵²

2.3.4 Monitoring kynurenine formamidase activity in mouse liver homogenates

An interactive program between personnel of the Biology Division and the Chemical Technology Division is underway to demonstrate the utility of the CFA in monitoring genetic differences in mice. Preliminary studies performed by personnel in the Biology Division had suggested that the activity levels of kynurenine formamidase (KF) might be indicative of genetic differences in mice. The reaction, illustrated in Eq. (70), is based on monitoring the rate of increase of kynurenine at 365 nm.

Table 2.29. Preliminary screening for serum enzymes

Sample	Enzyme activity (IU/liter at 30°C)		
	GGTP	LAP	5'-Nase
Normal female	0-33.3 ^a	15.0-28.6 ^a	≤20
Abnormal patients ^b			
125	834.8 (E) ^c	104.3 (E)	33.0 (E)
2082	117.2 (E)	29.5 (E)	ND ^d
2083	7.2	24.0	ND
2086	12.3	34.2 (E)	ND
2087	15.5	23.4	ND
2088	10.7	17.0	ND
2089	9.0	9.6	ND
2090	162.3 (E)	34.8 (E)	ND
2027	67.5 (E)	51.6 (E)	ND
2028	36.4 (E)	41.4 (E)	ND
2031	27.7	21.5	ND
2032	21.2	16.9	ND
2123	32.8	34.2 (E)	ND
2126	19.4	28.5	ND
2127	11.2	26.8	ND
1968	112.6 (E)	47.0 (E)	ND
1972	21.2	16.9	ND
2029	9.0	21.3	ND
1971	11.8	17.2	ND
1970	24.9	20.7	ND
2036	50.4 (E)	32.7 (E)	ND
1969	96.1 (E)	81.5 (E)	39.4 (E)

^a $\bar{x} \pm 2\sigma$.^bSamples provided by National Cancer Institute.^cActivity exceeds upper limit of normal range.^dNot detectable (<8 IU/liter).

Liver formamidase activities have been studied in 16 inbred strains of mice on the CFA. Nine of these show high activity, from 27 to 37 IU per gram of liver tissue, whereas seven others show low activity, ranging from 9 to 11 IU per gram. There are no intermediate activities among inbred strains, but F_1 hybrids of high and low inbred strains have activities that are intermediate between the two parental strains. This pattern of activity suggests that a single gene controls the level of formamidase activity in mice. The genetics of this system is still under investigation.

Using the CFA to monitor individual fractions from chromatographic columns, it was determined that there are at least two separable enzyme activities. One of the chromatographic peaks appears to be similar with respect to both quantity and chromatographic elution behavior in both high- and low-activity inbred strains, but a second peak, which accounts for the bulk of the formamidase activity, is very different in the two strains. Whether this difference is due to a variation in the hormonal regulation of a single enzyme or to the presence of isoenzymes remains to be determined. The formamidase activity in both strains was shown to increase 10- to 15-fold during development from three days before birth to the adult level. The adult levels of activity are reached at $\sim$ 12 days of age in the low strain and 24 days in the high one. Studies on this enzyme system are continuing in conjunction with personnel of the Biology Division.

2.3.5 Adaptation of Coombs testing to the Centrifugal Fast Analyzer

In the previous report² on the adaptation of Coombs testing to the miniature CFA, we showed that some samples characterized as being low-

level positive, by manual Coombs test procedures, were not differentiated from negative reactions when tested on the CFA. Based on initial results, additional experiments were performed with weakly agglutinating samples using a different technique to obtain absorbance measurements.

In preparing for these experiments, it was noted that the rotor used for Coombs testing was becoming more difficult to clean. Calibration of the rotor was becoming questionable, and aspiration of wash solutions from several cuvetts was inefficient. Upon disassembly, it was found that the rotor body was distorted, which prevented proper sealing of upper and lower windows. A crack between one of the reagent and sample cavities was also noted. Consequently, a new rotor was fabricated. There were no changes in the design of the rotor for these studies.

Background. The Coombs test or antiglobulin⁵³ reaction detects globulin, a protein component of serum, immunologically bound or coated on the surface of red blood cells. Red cells that have absorbed immunoglobulin molecules can be washed free of unattached globulin and other serum protein, but the antibody globulin will remain fixed to the antigens on the cell surface. When antihuman serum is added to washed, sensitized cells, the antiglobulin molecules form bridges between the globulin molecules. The end result is agglutination (clumping together of the cells) of the red cells. Therefore, the criterion for most blood banking procedures capable of demonstrating the antigen-antibody reaction in vitro is the presence or absence of hemagglutination.

Summary of previous results. Some of the major factors necessary for antiglobulin testing have previously been evaluated on the CFA using a rotor in which cell suspensions were washed in situ and hemagglutination was measured by changes in light transmission. These studies have confirmed

that aspiration of wash solutions is best accomplished, in a reproducible manner, when a rotational rate of 600 rpm is used. The importance of detecting all levels of globulin binding has also been indicated.

Previous studies involved the incubation of washed cells and antiserum for 5 min at 30°C while rotating slowly and measurement of the absorbance at 520 nm; this procedure was repeated twice. This method resulted in excessive time for analysis, and results were not entirely consistent, especially with respect to the identification of low-level positive reactions and their differentiation from negative reactions.

Kinetic sedimentation rate. Since we are dealing with reactions in which the degree of cell agglutination differs, monitoring of differential cell sedimentation in the centrifugal field was considered as a possible alternative analytical method. Differing degrees of cell agglutination should be characterized by differing rates of particulate sedimentation from the optical path of the CFA. Light transmission was monitored as a function of time for constant cell masses sensitized to varying degrees with Rh₀ (D) positive cells to test this hypothesis. Sedimentation was monitored by light transmission measurements at 2-sec intervals over a 60-sec time period. Results, shown in Fig. 2.40, indicated that differences in the rates of sedimentation were insignificant between low-level positive and negative reactions. Cells having higher antigen levels were sedimented so rapidly that sedimentation was, in the time domain of this analyzer, instantaneous with essentially no change in light transmission occurring during the monitored time domain. It is possible that optical measurements initiated much earlier in time can measure sedimentation rates which will detect low antigen levels. The measurements shown in Fig. 2.40 were

Fig. 2.40. Effect of cell sedimentation in the performance of direct Coombs testing with the Centrifugal Fast Analyzer (centrifugal field = $16.5 \times g$). Upper curve - negative reaction; lower curves - positive reactions, graded 4+ to $\pm$.

acquired on the miniature CFA, and ~ 7 sec elapsed before the first measurement was taken. The new portable CFA could start acquiring data within ~ 0.4 sec, thus enabling measurement of early sedimentation which may be more nearly proportional to antigen concentration.

Description of new assay procedure. Although differences in sedimentation rates were not significant, it was noted that differences in the earliest measurements of light transmission were proportional to the degree of cell sensitization. Based on these findings, a series of experiments was planned to study differential absorbance as a means of classifying antigenicity based on the earliest measurements of light transmission.

Antiserum titers were prepared in ratios of 1:1, 1:5, 1:25, 1:50, 1:75, and 1:100 by diluting anti-Rh₀ (D) antiserum in isotonic saline. Rh₀ (D) positive blood was drawn (from a donor) into vials containing ethylenediaminetetraacetic acid (EDTA) anticoagulant. The blood was washed four times with isotonic saline in a Sorvall cell washer. One milliliter of washed cells was then added to 1-ml aliquots of each dilution of the original antiserum titers, resulting in the preparation of Coombs positive cells of varying antigenic strengths but with a constant cell mass.

Six microliters of individual titer samples, whole blood, and Coombs control cells were then discretely loaded sequentially, in duplicate, into the sample cavities of the rotor with 50 μ l of saline diluent. Simultaneously, an additional 60 μ l of saline was added to each reagent cavity of the rotor. The rotor was then placed in the analyzer and accelerated to 3000 rpm and braked. The accelerate/brake

process was repeated three times to facilitate mixing. Next, the rotor was accelerated to 4000 rpm for 60 sec and slowed, without braking, to 600 rpm; the wash solution was aspirated; and 130 μ l of saline per cuvet was loaded dynamically while rotating at 2500 rpm. The procedure was repeated three times to ensure adequate washing of cells. The rotor containing the washed cells was then returned to the rotor loading station, and 10 μ l of antihuman serum plus 50 μ l of saline were loaded into each cavity. Fifty microliters of 4% polyvinylpyrrolidone (PVP), a polyelectrolyte used to enhance agglutination, and 6 μ l of saline diluent were added to the reagent cavities of the rotor. The rotor was again placed in the analyzer, and the temperature was adjusted to 30°C with the rotor spinning at about 50 rpm. Upon reaching 30°C, the rotor was twice accelerated to 4000 rpm and braked to facilitate mixing of the sensitized blood cell samples and the antiserum. The reaction mixture was then incubated at 30°C for 5 min while rotating slowly. After incubation, the rotor was sequentially accelerated to 4000 rpm for 45 sec, braked, and then accelerated to 3000 rpm and braked two successive times. This dynamic braking from 3000 rpm to stop in a fraction of a second resuspends the nonagglutinated but not the agglutinated cells.⁵⁴ Figure 2.41 schematically illustrates the mechanisms of this process from incubation through centrifugation to resuspension. Rotational speed of the rotor was then adjusted to 600 rpm, and the absorbance was determined immediately at 520 nm.

Results. The initial absorbance (representative of sedimentation) of nonsensitized (whole blood) cells is vastly different from that of sensitized Rh₀ (D) positive cells. Data were collected by taking four measurements of light transmission through the cuvetts at 2-sec intervals.

Fig. 2.41. Schematic illustration of the monitoring process on the Centrifugal Fast Analyzer for the presence or absence of the hemagglutination reaction in the Coomb's test.

The weakly sensitized cells form small dispersed agglutinates which sediment more rapidly than nonsensitized cells, thus resulting in more highly transmitting solutions. Evidence of this is shown in Fig. 2.42 where the initial absorbances (averages of the first three measurements) of eight different suspensions are plotted vs the antigen dilution. The order of dilution (antiserum to saline ratio) in these titers shows a direct relationship between antigenicity and the degree of agglutination. The highest absorbance represents a nonsensitized (whole blood) cell sample. These results are also shown in Table 2.30 where it can be seen that no agglutination is observed for nonsensitized (whole blood) cells and the reaction grading (as described in the previous report²) is negative, whereas the Coombs control cells exhibit extremely large clumps of cells with almost all cells agglutinated and have a very strong 4+^S reaction grading. The prepared titers fall between these two extremes and range from very fine clumps to very large clumps of agglutinated cells. Reaction grading ranges from very low globulin binding levels to very strongly bound levels ($\pm$ to 4+^S in Table 2.30). Our data from these experiments suggest that we can define an agglutination reaction with an absorbance > 1.0 as a negative reaction and a reaction with an absorbance < 1.0 as a positive reaction.

These experiments characterize only the D antigen, which distinguishes Rh positive and Rh negative persons.⁵³ There are other antigens recognized in the Rh system which also elicit an agglutination response to foreign antibody production. The major antigens are C, c, E, and e, and these may produce levels of globulin binding equivalent to or lower than those observed for the D antigen. Therefore, the limits between positive and negative reactions, as defined from these experiments, may be modified

Fig. 2.42. Illustration of absorbance measurements obtained for various concentrations of antigen showing the relatively large difference between even the weakest level of antigen ($\pm$ visual reaction grading) and the known negative serum.

Table 2.30. Automated direct Coombs "antiglobulin" test^a in a miniature Centrifugal Fast Analyzer

ANTI-Rh _C (ANTI-D) TITER (DILUTION)	0 ^b	1:10C	1:75	1:50	1:25	1:5	1:1	COOMBS CONTROL CELLS
MEAN ABSORBANCE (520 nm)	1.397	0.5912	0.2826	0.3472	0.2112	0.0596	0.0483	0.0088
DEGREE OF AGGLUTINATION	NO VISIBLE AGGLUTINATION	VERY FINE AGGLUTINATION AMONG NUMEROUS FREE CELLS	NUMEROUS TINY CLUMPS	MANY SMALL CLUMPS	MANY MEDIUM SIZED CLUMPS	MANY LARGE CLUMPS	MANY VERY LARGE CLUMPS	EXTREMELY LARGE CLUMPS ALMOST NO FREE CELLS
REACTION GRADING	(-) 0	+ -	1+	2+	2+ ^s	3+	4+	4+ ^s

^a PROCEDURE:

1. SAMPLE; 6-μl CELL SUSPENSION + 10-μl ISOTONIC SALINE.
2. IN SITU WASHING 3 TIMES WITH ISOTONIC SALINE.
3. 10-μl COOMBS ANTIGLOBULIN SERUM + 50-μl POLYVINYLPIRROLIDONE + 50-μl ISOTONIC SALINE.
4. FIVE-MINUTE INCUBATION AT 30°C.
5. CENTRIFUGE, DISPERSE, MEASURE ABSORBANCE AT 520 nm.

TITER: 1 PART ANTI-Rh₀ (ANTI-D) SERUM TO 1, 5, 25, 50, 75, and 100 PARTS ISOTONIC SALINE, INCUBATED 1 HOUR AT 30°C. EQUAL VOLUMES OF INDIVIDUAL DILUTIONS ADDED TO WASHED Rh₀ POSITIVE (D POSITIVE) CELLS RESULTING IN VARYING ANTIGEN STRENGTHS WITH CONSTANT CELL MASS.

^b WHOLE BLOOD.

and defined more accurately as samples are assayed for other antigens and compared with the limits of manual methods of grading.

Comparisons of the six titers, Coombs control cells, and whole blood are shown in Figs. 2.43 and 2.44. These samples (the same as those run on the miniature CFA) were subjected to manual Coombs test procedures for this comparison and are shown magnified 250X. The rotor used for these experiments has the capability of allowing microscopic examination of questionable hemagglutination reactions simply by removing reactant products directly from the cuvet in question, depositing the reaction mixture on a slide, and examining it microscopically (see Fig. 2.45 for comparison of Coombs test rotor and standard rotor).

Conclusions. The results obtained from the experiments performed during this report period clearly indicate that low levels of globulin binding can be detected by light transmission methods on the miniature CFA. These experiments served to characterize only the D antigen; additional antigens and samples classified by manual techniques from hospitalized patients must be assayed. At this time, the use of the miniature CFA for monitoring the antigen-antibody reaction is feasible for the D antigen. The results of continued efforts in the adaptation of Coombs testing to the CFA could yield an important assistance to blood banking procedures.

2.3.6 Environmental analysis

Analysis of sulfate ion. An ongoing project in cooperation with both the Analytical Chemistry Division and the Environmental Sciences Division at ORNL involves the determination of trace quantities of sulfate ion (SO_4^{2-}) in water. The limitations of existing methodologies

COOMBS CONTROL CELLS
REACTION GRADE = 4 +^s

SENSITIZED Rho (D) CELLS 1:1
REACTION GRADE = 4 +

SENSITIZED Rho (D) CELLS 1:5
REACTION GRADE = 3 +

SENSITIZED Rho (D) CELLS 1:25
REACTION GRADE = 2 +^s

Fig. 2.43. Degree of agglutination resulting from manual direct Coombs testing.

SENSITIZED Rho (D) CELLS 1:50
REACTION GRADE = 2 +

SENSITIZED Rho (D) CELLS 1:75
REACTION GRADE = 1 +

SENSITIZED Rho (D) CELLS 1:100
REACTION GRADE = $\pm$

UNSENSITIZED Rho (D) CELLS
REACTION GRADE = 0 (NEG.)

Fig. 2.44. Degree of agglutination resulting from manual direct Coombs testing.

Fig. 2.45. Comparison of a standard Centrifugal Fast Analyzer rotor (a) with the rotor designed for washing of cells in situ (b).

and the possibility of a novel, direct, kinetic spectrophotometric method for use with the miniature CFA have been reported previously.³ The method, based on the catalytic effect of sulfate ion on the zirconium-methylthymol blue reaction,⁵⁵ has been further evaluated for use in the analysis of environmental samples.

One difficulty in the evaluation of the kinetic data is that the time interval for which the reaction rate is apparently first-order for the sulfate ion varies with the age of the zirconium reagent (occurring at longer intervals as the zirconium ages and becoming more high polymerized). In addition, within a run the linear segment of the reaction progress curve occurs at shorter time intervals as the concentration of sulfate ion is increased. In order to automate the selection of the time interval used for computation of reaction rate, a linear search program (analogous to that described previously for the determination of CPK isoenzymes) is used. A typical computer output utilizing this linear search subroutine is presented in Table 2.31. The minimum acceptable correlation coefficient selected (0.9993) permits discrimination against the initial rapid increase in absorbance due to the presence of nonpolymerized zirconium in the reagent. Following this initial rapid increase, the reaction is governed by the rate of depolymerization catalyzed by the sulfate ion.³ This permits a single set of observation conditions to be used for a variety of sample concentrations and reagent ages.

The precision in the determination of reaction rate using the linear search subroutine is 1 to 2%, or 0.2 to 0.3 ppm SO_4^{2-} . The detection limit (two times the standard deviation of the rate measurement of a 2 ppm SO_4^{2-} standard, divided by the slope of the calibration curve) is ~ 0.3 ppm. Run-to-run reproducibility (Table 2.32) and day-to-day reproducibility (Table 2.33) are both of the order of ± 0.2 ppm SO_4^{2-} .

Table 2.31. Good reproducibility is demonstrated for the measurement of sulfate on the Centrifugal Fast Analyzer by using the linear search computer program to overcome the problem of initial reaction nonlinearity

RUN NUMBER: 3,
 NUMBER OF CUVETS UNDER CONSIDERATION: 17,
 GEMSAEC UNIT: 17.2,
 DELAY INTERVAL: 100,
 OBSERVATION INTERVAL: 30,
 NUMBER OF SETS OF OBSERVATIONS: 30,
 MINIMUM ACCEPTABLE CORRELATION COEFFICIENT: 0.9993,

CUVET	INTERVAL (SEC)	SO_4^{2-} Conc.	RATE (A/MIN)	CC
2	280 - 730	0 ppm	0.020339	0.999620
3	280 - 730		0.020260	0.999530
4	250 - 700	5 ppm	0.024149	0.999428
5	250 - 700		0.024459	0.999391
6	220 - 670	10 ppm	0.028198	0.999355
7	220 - 670		0.028117	0.999367
8	220 - 670	15 ppm	0.031905	0.999488
9	220 - 670		0.032066	0.999407
10	190 - 640	20 ppm	0.036377	0.999476
11	190 - 640		0.036486	0.999581
12	250 - 700	Sample #1	0.024886	0.999544
13	250 - 700		0.024344	0.999585
14	250 - 700		0.024938	0.999488
15	220 - 670	Sample #2	0.038488	0.999478
16	220 - 670		0.038599	0.999571
17	220 - 670		0.037926	0.999471

Table 2.32. Run-to-run reproducibility in sample analysis

Sample	Run 1	Run 2	Run 3	Run 4	Run 5	Average
1	2.9 ± 0.1	2.6 ± 0.4	2.3 ± 0.1	2.3 ± 0.1	2.2	2.6 ± 0.3
2	3.1 ± 0.1	3.0 ± 0.3	3.1 ± 0.1	2.7 ± 0.1	3.6 ± 0.0	3.1 ± 0.3
3	5.3 ± 0.1	5.3	5.3 ± 0.3	5.1	5.5 ± 0.3	5.3 ± 0.1
4	20.9 ± 0.0	21.0 ± 0.2	20.1 ± 0.2	21.4 ± 0.2	21.3 ± 0.4	20.9 ± 0.5

Table 2.33. Day-to-day reproducibility in sample analysis

Day	Sample No.			
	1	2	3	4
1	2.7 ± 0.3	2.9 ± 0.2	5.6 ± 0.1	21.1 ± 0.2
2	2.9 ± 0.7	3.1 ± 0.3	5.4 ± 0.3	21.4 ± 1.3
3	2.6 ± 0.3	3.1 ± 0.3	5.3 ± 0.2	20.9 ± 0.5
4	3.2 ± 0.4	3.4 ± 0.3	5.5 ± 0.3	21.3 ± 0.4
Average	2.8 ± 0.3	3.1 ± 0.2	5.4 ± 0.1	21.2 ± 0.2

Cationic interferences are eliminated by treating 0.5-ml aliquots of the sample batchwise with ~ 100 mg of Amberlite 120 ion exchange resin (sodium form) prior to analysis. Hems *et al.*⁵⁵ evaluated the effect of diverse anionic interferences upon the reaction rate. Fluoride (F^-), phosphate (PO_4^{3-}), and arsenate (AsO_4^{3-}) were found to interfere with the determination of the sulfate ion, even when present in trace concentrations. Hems found that interference by these anions could be eliminated by boiling the sample with excess magnesium oxide, followed by filtration of the sample.⁵⁵ We have found that F^- , PO_4^{3-} , and AsO_4^{3-} could be masked more expeditiously by the addition of 150 λ of 200 ppm $AlCl_3$ to 1.0 ml of the cation exchange-treated sample. Without masking, 1 ppm concentrations of F^- , PO_4^{3-} , and AsO_4^{3-} are equivalent to 15, 9, and 2 ppm SO_4^{2-} , respectively. In contrast, with masking, concentration of these anions at the 2 ppm level (exceeding the upper limit of these species found in surface waters⁵⁶) produces errors of ≤ 0.25 ppm (as SO_4^{2-}). Sulfate recovery in samples subjected to treatment with ion exchange resin and aluminum ion (Al^{3+}) averaged 101%.

A preliminary comparison between the present technique and a reference method [based on the determination of the residual barium ion (via complexation with methylthymol blue) after precipitation of sulfate as barium sulfate^{57,58}] is presented in Table 2.34. The reference method was performed independently by the Environmental Analysis Section, Analytical Chemistry Division, ORNL, and the samples are rainwater and double-blind control samples provided by the Environmental Sciences Division, ORNL. The agreement of the two methods performed in different laboratories is quite excellent for such low concentrations of sulfate ion. A major

Table 2.34. Correspondence of the Centrifugal Fast Analyzer with a reference method for the determination of sulfate ion

Sample number	SO_4^{2-} found, ppm	
	Technicon Autoanalyzer (X)	CFA kinetic (Y)
1	2.8	2.8
2	2.8	3.1
3	21.0	21.2
4	2.8	3.1
5	1.4	1.6
6	3.5	3.3
7	4.0	4.2
8	2.5	2.2
9	1.4	1.0
10	3.5	2.7
11	6.3	5.8

Linear least-squares regression analysis:

slope = 1.01 ± 0.02

Y intercept = -0.15 ± 0.47 ppm

correlation coefficient = 0.9979

std error of estimate = 0.35 ppm

student's T = 46

disadvantage of the reference technique, based upon formation of sparingly soluble BaSO_4 , is that deposits of salt coat the flow cell of the instrument during a run, causing a baseline shift and a shift of the calibration curve.^{56,57} Other difficulties include sample carry-over by the Auto-Analyzer ($\geq 6\%$), difficulty in achieving mixing of sample and reagents, and oxidation of methylthymol blue indicator in a basic medium. In contrast, the kinetic method does not involve the formation of BaSO_4 . Samples and reagent are loaded discretely, and methylthymol blue reagent is in an acidic medium in which it is stable for at least 4 months. Also, since a kinetic procedure is utilized, there is minimal interference due to the sample blank absorbance.

Extraction, analysis, and interpretation of adenosine triphosphate in environmental samples. The adaptation to the CFA of a hexokinase enzymatic assay for the determination of ATP was introduced in a previous report.³ This assay has been evaluated extensively by the Environmental Sciences Division, ORNL, in order to test extraction procedures for ATP in a variety of substrates. On the basis of these studies, a chloroform method (see Table 2.35) has been selected as most suitable for routine use. In the chloroform treatment, ATP is partitioned into the buffer phase, which is subsequently separated. Treatment of this phase with carbon tetrachloride (CCl_4) (immiscible) serves two functions: (1) residual chloroform (CHCl_3) is removed (CHCl_3 is soluble to the extent of 1% in water and can interfere in subsequent enzymatic reactions), and (2) the sample is further decolorized by the removal of organic material.

Table 2.35. Chloroform method for the extraction
adenosine triphosphate from
environmental samples

I. Chloroform treatment

1 g (wet weight) of sample
+ 6 ml Tris buffer, pH 7.4
+ 3 ml CHCl_3

Vortex mixture, then sonicate for 30 sec.
Centrifuge 10 min at 1000 x g.

II. Treatment with carbon tetrachloride

Withdraw 5 ml of buffer phase above.
Add 2 ml CCl_4 , and vortex.
Centrifuge 1 min at 1000 x g.
Buffer phase is used for assay of ATP.

In Table 2.36, three extraction procedures and two chemical assays are compared for the analysis of ATP extracted from various substrates. The spectrophotometer hexokinase assay was performed on a simple, inexpensive instrument without correction for sample blank. Thus, the method is subject to error when the test sample has a high initial absorbance. The methodological difficulties involved in the reference luciferin-luciferase chemiluminescence method have been described previously.³ In general, the chloroform method is superior to treatment with butanol or H_2SO_4 for the extraction of ATP, and agreement is satisfactory between the reference method and the CFA hexokinase method. Agreement between the CFA hexokinase method (with automatic sample blanking) and the manual spectrophotometric method is best for the $CHCl_3$ extraction, which minimizes sample blank absorbance.

In addition to the determination of ATP, it is hoped that total "adenylate energy charge" can be measured. The adenylate energy charge is a function of the concentration of ATP, ADP, and AMP and has been proposed to be a better index of microbial activity than is ATP concentration alone.^{6,59}

Table 2.36. Portion of adenosine triphosphate detected using three extraction procedures and two chemical assays

Substrate	Extractant	$\bar{x}$ % ATP CONCENTRATION DETECTED(S.E.) ^a		
		Liquid Scintillation	Centrifugal Fast Analyzer	Spectrophotometer ^b
		Luciferin-Luciferase	Hexokinase	Hexokinase
Stream water	H ₂ SO ₄	79.5(5.5)	82.3(4.6)	56.5(10.7)
	Butanol	89.6(3.6)	100.0(5.0)	186.0(18.6)
	Chloroform	93.6(2.1)	108.0(3.1)	173.0(12.3)
Sand-Kaolinite	H ₂ SO ₄	86.9(6.8)	75.0(7.5)	62.9(17.3)
	Butanol	83.6(9.3)	100.0(6.3)	188.0(30.9)
	Chloroform	81.7(3.4)	96.7(5.5)	98.0(10.1)
Leaf litter	H ₂ SO ₄	137.0(31.0)	177.0(26.1)	300.0(23.6)
	Butanol	81.1(18.1)	73.9(15.3)	44.4(10.1)
	Chloroform	92.3(9.6)	86.8(10.1)	85.3(8.3)
Pasture soil	H ₂ SO ₄	69.8(3.9)	82.1(1.9)	59.5(5.9)
	Butanol	65.7(8.6)	37.5(10.1)	84.0(4.0)
	Chloroform	92.4(3.9)	89.1(4.6)	81.3(5.8)
Forest soil	H ₂ SO ₄	130.0(12.9)	119.0(6.5)	183.0(20.5)
	Butanol	89.6(9.1)	80.2(8.1)	154.0(12.3)
	Chloroform	93.5(3.7)	103.0(4.8)	123.0(10.6)
Sawdust	H ₂ SO ₄	35.1(10.5)	28.0(11.1)	103.0(12.6)
	Butanol	76.9(8.3)	95.5(7.3)	374.0(27.3)
	Chloroform	91.0(6.1)	85.3(10.1)	86.9(10.7)

^aRelative standard error in parentheses.^bBausch & Lomb Spectrometer 20. No sample blank was used.

2.4 References for Section 2

1. C. A. Burtis, W. D. Bostick, and W. F. Johnson, "Development of a Multipurpose Optical System for Use with a Centrifugal Fast Analyzer," Clin. Chem. 21, 1225 (1975).
2. J. E. Mrochek, C. A. Burtis, and C. D. Scott, Biochemical Technology Program Progress Report for the Period January 1-June 30, 1976, ORNL/TM-5446 (September 1976).
3. C. A. Burtis, J. E. Mrochek, and C. D. Scott, Biochemical Technology Program Progress Report for the Period July 1-December 31, 1975, ORNL/TM-5167 (January 1976).
4. L. Bowie, F. Esters, J. Bolin, and N. Gochman, "Development of an Aqueous Temperature-Indicating Technique and Its Application to Clinical Laboratory Instrumentation," Clin. Chem. 22, 449 (1976).
5. D. C. Motors, Speed Controls, Servo Systems, 2nd ed., pp. 2-19, Electro-Craft Corp., Hopkins, Minnesota 55343.
6. R. B. McComb and G. N. Bowers, Jr., "Study of Optimum Buffer Conditions for Measuring Alkaline Phosphatase Activity in Human Serum," Clin. Chem. 18, 97 (1972).
7. D. Plaut and J. MacQueen, The Isoenzymes of Creatine Kinase (CPK), A Review, lit. no. CH-738, Dade Division American Hospital Supply Corporation, Miami, Florida, 1976.
8. S. A. Witteveen, B. E. Sobel, and M. DeLuca, "Kinetic Properties of the Isoenzymes of Human Creatine Phosphokinase," Proc. Nat. Acad. Sci. USA 71, 1384 (1974).
9. A. F. Smith, D. Radford, and C. P. Wong, "Creatine Kinase Isoenzyme Studies in Myocardial Infarction," Clin. Chem. 21, 974 (1975).
10. M. A. Varat and D. W. Mercer, "Cardiac Specific Creatine Phosphokinase Isoenzyme in the Diagnosis of Acute Myocardial Infarction," Circulation 51, 855 (1975).
11. P.C.-P. Wong and A. F. Smith, "Comparison of 3 Methods of Analysis of the MB Isoenzyme of Creatine Kinase in Serum," Clin. Chim. Acta 65, 99 (1975).
12. G. Lum and A. L. Levy, "Chromatographic and Electrophoretic Separation of Creatine Kinase Isoenzymes Compared," Clin. Chem. 21, 1601 (1975).
13. Helena Update, vol. 29, January 1976.
14. Worthington's World 4(4) (June 1976).
15. D. W. Mercer and M. A. Varat, "Detection of Cardiac-Specific Creatine Kinase Isoenzyme in Sera with Normal or Slightly Increased Total Creatine Kinase Activity," Clin. Chem. 21, 1088 (1975).

16. W. G. Yasmineh and N. Q. Hanson, "Electrophoresis on Cellulose Acetate and Chromatography on DEAE-Sephadex A-50 Compared in the Estimation of Creatine Kinase Isoenzymes," Clin. Chem. 21, 381 (1975).
17. L. G. Morin, "Improved Separation of Creatine Kinase Cardiac Isoenzyme in Serum by Batch Fractionation," Clin. Chem. 22, 92 (1976).
18. D. Mercer, "Poor Separation of Creatine Kinase Isoenzymes with Column Chromatographic Kits," Clin. Chem. 22, 552 (1976).
19. S. M. Sax, J. J. Moore, J. L. Giegel, and M. Welsh, "Atypical Increase in Serum Creatine Kinase Activity in Hospital Patients," Clin. Chem. 22, 87 (1976).
20. P. S. Rao, J. J. Lukes, S. M. Ayres, and H. Mueller, "New Manual and Automated Method for Determining Activity of Creatine Kinase Isoenzyme MB, by Use of Dithiothreitol: Clinical Applications," Clin. Chem. 21, 1612 (1975).
21. R. M. Balkcom, "Evaluation of the Chemical Activation Procedure (Rao's Method) for the Measurement of the MB Isoenzyme of Creatine Kinase," Clin. Chem. 22, 929 (1976).
22. M. Sheehan and K. Leipper, "Evaluation of the Chemical Activation Procedure (Rao's Method) for the Measurement of the MB Isoenzyme of Creatine Kinase," Clin. Chem. 22, 930 (1976).
23. B. K. Hultman and W. G. Yasmineh, "Evaluation of the Chemical Activation Procedure (Rao's Method) for the Measurement of the MB Isoenzyme of Creatine Kinase," Clin. Chem. 22, 932 (1976).
24. F. Muschenheim, "Evaluation of the Chemical Activation Procedure (Rao's Method) for the Measurement of the MB Isoenzyme of Creatine Kinase," Clin. Chem. 22, 932 (1976).
25. C. A. Burtis, J. C. Mailen, W. F. Johnson, C. D. Scott, T. O. Tiffany, and N. G. Anderson, "Development of a Miniature Fast Analyzer," Clin. Chem. 18, 753 (1972).
26. C. A. Burtis, W. F. Johnson, J. C. Mailen, J. B. Overton, T. O. Tiffany, and W. B. Watshy, "Development of an Analytical System Based Around a Miniature Fast Analyzer," Clin. Chem. 19, 895 (1973).
27. P. S. Rao and H. S. Mueller, "Evaluation of the Chemical Activation Procedure (Rao's Method) for the Measurement of the MB Isoenzyme of Creatine Kinase," Clin. Chem. 22, 932 (1976).
28. Worthington's World 4(3)(April 1976).
29. R. J. L. Bonder and G. A. Moss, Clin. Chem. 22, 554 (1976).
30. D. E. Burns, J. H. Ladenson, and J. E. Davis, "Lag Phase in Creatine Phosphokinase (CPK) Analysis," Clin. Chem. 21, 987 (1975).

31. D. M. Goldberg, G. Ellis, and A. R. Wilcock, "Problems in the Automation of Enzyme Assays with Lag, Accelerated and Blank Reactions," Ann. Clin. Biochem. 8, 189 (1971).
32. G. P. Hicks, R. A. Ziesemer, and N. W. Tietz, "The Use of Laboratory Computers in Monitoring Kinetic Enzyme Assays," Clin. Chem. 19, 27 (1973).
33. H. A. Laitinen, Chemical Analysis, pp. 538-45, McGraw-Hill, New York, 1960.
34. R. M. Bethea, B. S. Suran, and T. L. Boullion, Statistical Methods for Engineers and Scientists, pp. 137-45, Marcel Dekker, New York, 1975.
35. Calbiochem CPK/MB Reagent Package Insert (Feb. 1, 1976).
36. M. Hano, "The Detection of CPK₁ (BB) in Serum," Am. J. Clin. Pathol. 65, 351 (1976).
37. J. G. Batsakis, "Serum Enzymes and Cancer: Their Use in Hepatic Metastases," pp. 1-18 in Proceedings of 1st Invitational Symposium on the Serodiagnosis of Cancer, Armed Forces Radiobiology Research Institute, Bethesda, Maryland, 1974.
38. M. Flasher, "Biochemical Markers for Cancer," presented at the September 1976 meeting of the Capitol Section, American Association of Clinical Chemists.
39. C. D. Scott et al., p. 37 in Chemical Development Section B Semiannual Progress Report, March 1, 1973 to August 31, 1973, ORNL/TM-4370, (February 1974).
40. W. Nagel, F. Willig, and F. H. Schmidt, "Über die Aminosäurearylamidase- (sog-Leucinaminopeptidase-) Aktivität in Menschlichen Serum," Klin. Wochenschr. 42, 447 (1964).
41. T. Samorajski, C. Rolsten, and J. M. Ordy, "A Simplified Method for Determinations of Serum Leucine Aminopeptidase," Am. J. Clin. Pathol. 38, 645 (1972).
42. R. W. Phillips and E. R. Manildi, "Evaluation of Leucine Aminopeptidase in Disseminated Malignant Disease," Cancer 26, 1006 (1970).
43. S. Mizutani, M. Yoshino, and M. Oya, "Placental and Nonplacental Leucine Aminopeptidases During Normal Pregnancy," Clin. Biochem. 9, 16 (1976).
44. G. Szasz, "A Kinetic Photometric Method for Serum Leucine Aminopeptidase," Am. J. Clin. Pathol. 47, 607 (1967).
45. D. M. Goldberg, "5'-Nucleotidase: Recent Advances in Cell Biology, Methodology, and Clinical Significance," Digestion 8, 87 (1973).

46. W. van der Slik, J.-P. Persign, E. Engelsman, and A. Riethorst, "Serum 5'-Nucleotidase," *Clin. Biochem.* 3, 59 (1970).
47. J.-P. Persign, W. van der Slik, K. Kramer, and C. A. de Ruijter, "A New Method for the Determination of Serum Nucleotidase," *Z. Klin. Chem. Klin. Biochem.* 6, 441 (1968).
48. A. Belfield, G. Ellis, and D. M. Goldberg, "A Specific Colorimetric 5'-Nucleotidase Assay Using Berthelot Reaction," *Clin. Chem.* 16, 396 (1970).
49. G. Ellis and D. M. Goldberg, "An Improved Kinetic 5'-Nucleotidase Assay," *Anal. Lett.* 5, 65 (1972).
50. J. Bootsma, B. G. Wolthers, and A. Groen, "Determination of Serum 5'-Nucleotidase by Means of a NADH-Linked Reaction," *Clin. Chim. Acta* 41, 219 (1972).
51. A. Belfield and D. M. Goldberg, "Application of a Continuous Spectrophotometric Assay for 5'-Nucleotidase Activity in Normal Subjects and Patients with Liver and Bone Disease," *Clin. Chem.* 15, 931 (1969).
52. O. D. Kowlessar, L. J. Haeffner, E. M. Riley, and M. H. Sleisenger, "Comparative Study of Serum Leucine Aminopeptidase, 5'-Nucleotidase, and Nonspecific Alkaline Phosphatase in Diseases Affecting the Pancreas, Hepatobiliary Tree, and Bone," *Am. J. Med.* 31, 231 (1961).
53. Blood Group Antigens and Antibodies as Applied to the ABO and Rh Systems, Ortho Diagnostics, Inc., Raritan, New Jersey (1969).
54. T. O. Tiffany, J. M. Parella, C. A. Burtis, W. F. Johnson, and C. D. Scott, "Blood Grouping with a Miniature Centrifugal Fast Analyzer," *Clin. Chem.* 20, 1043 (1974).
55. R. V. Hems, G. F. Kirkbright, and T. S. West, "Kineto-chromic Spectrophotometry - II. Determination of Sulfate by Catalysis of the Zirconium-Methylthymol Blue Reaction," *Talanta* 16, 789 (1969).
56. R. G. Bond and C. P. Straub (eds.), Handbook of Environmental Control. Vol. III: Water Supply and Treatment, p. 22, CRC Press, Cleveland, Ohio, 1972.
57. A. L. Lazrus, K. C. Hill, and J. P. Lodge, "A New Colorimetric Micro-determination of Sulfate Ion," pp. 291-93 in Automation in Analytical Chemistry, Technicon Symposia, 1965.
58. M. R. McSwain, R. J. Watrous, and J. E. Douglass, "Improved Methylthymol Blue Procedure for Automated Sulfate Determination," *Anal. Chem.* 46, 1329 (1974).
59. T. M. Ching and K. K. Ching, "Content of Adenosine Phosphates and Adenylate Energy Charge in Germinating Ponderosa Pine Seeds," *Plant Physiol.* 50, 536 (1972).

3. ADVANCED ANALYTICAL SYSTEMS

W. M. Ayers*, J. S. Eady**, R. K. Genung, N. E. Lee
J. E. Mrochek, and J. T. Walsh***

Several analytical systems employing high-resolution chromatographic (HRLC) separations are being used on a routine basis to analyze specific molecular constituents of physiologic fluids. In addition, a system for the automated fractionation of whole blood into plasma, washed erythrocytes, and hemolysate fractions is under development. Techniques and instrumentation described are designed to yield analytical results for specific molecular constituents to enable correlation with clinical diagnoses of patients' conditions or to automate time-consuming laboratory procedures.

3.1 High-Resolution Liquid Chromatographic Systems

We are continuing our studies of the application of HRLC to the analysis of protein-bound carbohydrates in human serum as potential biochemical markers of cancer. A new area of application for HRLC is the separation of isoenzymes with direct monitoring of the column eluent by fluorescence photometry.

3.1.1 Chromatographic analysis for protein-bound carbohydrates in normal female subjects and breast-cancer patients

Previous studies^{1,2} had indicated that protein-bound fucose in the sera of patients with breast cancer seemed to mirror the state of the malignancy. That is, decreasing fucose signaled patient improvement, whereas increasing fucose signaled progressive disease. Protein-bound sialic acid, on the other hand, went through some wild fluctuations in concentration, but its response to the clinical state of the patients' malignancies did not seem uniform. There was some question concerning the results for sialic acid since these assays were performed on serum

*Summer Employee, MIT Practice School.

**Great Lakes College Association Student.

***Exceptional Student Program.

samples which had been previously thawed for assay of protein-bound mannose, galactose, and fucose. Studies were continued in this area with sera being assayed for all four protein-bound carbohydrates after being thawed the first time.

Protein-bound carbohydrates in sera from normal females. Sera from 20 normal female subjects were assayed for protein-bound mannose, galactose, fucose, and sialic acid by procedures which have been described previously.¹⁻³ Mean results and 95% confidence limits are illustrated in Table 3.1.

Table 3.1. Mean and 95% confidence limits for protein-bound mannose, galactose, fucose, and sialic acid in sera from normal women

mg/liter of serum			
Sialic acid	Fucose	Mannose	Galactose
387($\pm$ 137) ^a	45.5($\pm$ 27.2) ^b	480($\pm$ 133) ^c	393($\pm$ 157) ^c

^aMean and 95% confidence limits for 20 samples.

^bMean and 95% confidence limits for 49 samples.

^cMean and 95% confidence limits for 54 samples.

Results for fucose, mannose, and galactose are very similar to those obtained for previous normals; however, the normal range for sialic acid was considerably lowered. Illustrated in Fig. 3.1 are the individual results for these 20 normal women; the mean and 95% confidence limits ($\pm 2\sigma$) for 50 samples are shown for mannose, galactose, and fucose, whereas the mean shown for sialic acid represents only the 20 samples illustrated in the figure.

ORNL DWG 76-14791

Fig. 3.1. Serum protein-bound carbohydrates in normal nonfasting women.

One of the two samples having a fucose level greater than $+2\sigma$ from the mean also had the highest assays for mannose and galactose and had a $+1\sigma$ level for sialic acid. This subject was 49 years of age; however, nothing further is known about her.

Protein-bound carbohydrates in sera from patients with breast cancer.

Sera from 24 patients with breast cancer were assayed for the four protein-bound carbohydrates, mannose, galactose, fucose, and sialic acid. These patients have been examined clinically, and the status of their malignancy has been determined and noted. This information is not currently available since we are performing blind assays of the samples; however, when the entire series of samples has been completed, the disease status will be correlated with assay results.

Illustrated in Table 3.2 are assays for the four protein-bound carbohydrates in sera from breast-cancer patients. Note that 92% of these patients manifested both fucose and sialic acid in excess of the 95% confidence limits for normal women. High levels of mannose and galactose were only observed in 42 and 33% of the cases, respectively. The incidence of elevated sialic acid is considerably higher than we had reported previously;² however, the mean normal data are also considerably lower than our previous data on multiply-thawed samples (680 mg/liter). As we had determined earlier, neither mannose nor galactose seems to be useful as a marker of malignancy.

3.1.2 High-resolution chromatographic separation of isoenzymes

Recently a new, small-particle-size separations medium has become available. This material, a 5- to 10- μ m-sized fraction of controlled pore glass (CPG) from Corning, was available with a thin layer of a

Table 3.2. Protein-bound carbohydrates in sera from patients with breast cancer

Protein-bound carbohydrates, mg/liter serum				
Sample no.	Mannose	Fucose	Galactose	Sialic acid
361	542 ^a	112 ^a	536	519
391	562 ^a	99.2 ^a	558 ^a	722 ^a
521	367	103	323	560 ^a
645	440	69.4	430	557 ^a
678	472	88.0 ^a	377	636 ^a
1015	477	89.0 ^a	444	743 ^a
1063	509	84.4 ^a	463	615 ^a
1140	546 ^a	98.8 ^a	499	1013 ^a
1232	468	115	416	656 ^a
1235	524	73.6	488	668 ^a
1271	738 ^a	134 ^a	664 ^a	1298 ^a
1439	679 ^a	114 ^a	673 ^a	1585 ^a
1463	498	91.6 ^a	523	711 ^a
1508	996 ^a	202 ^a	1011 ^a	1493 ^a
1509	608 ^a	123 ^a	627 ^a	1059 ^a
1524	493	127 ^a	460	770 ^a
1599	416	83.0 ^a	388	704 ^a
1624	491	129 ^a	474	836 ^a
1627	456	145 ^a	471	669 ^a
1761	635 ^a	134 ^a	638 ^a	1004 ^a
1853	675 ^a	206 ^a	687 ^a	926 ^a
2047	507	140 ^a	478	769 ^a
2072	659 ^a	142 ^a	591 ^a	884 ^a
2253	407	130 ^a	375	594

^aGreater than +2 σ from the mean.

glycerol polymer bonded to the surface of the CPF (glycophase) and ion exchange groups coupled to this hydrophilic polymer skin (diethylamino-ethanol, DEAE). This material was reported to separate the isoenzymes of lactate dehydrogenase with excellent resolution in < 1 hr.⁴

We were interested in CPG-DEAE glycophase as a separations medium for an automated assay system which would incorporate direct monitoring of the column effluent for enzyme activity. Several factors led us to select the creatine phosphokinase (CPK) isoenzyme system for our initial development studies. In terms of clinical utility, elevated serum levels of the CPK-MB isoenzyme have been shown to be diagnostic for myocardial infarction.⁵⁻⁸ In addition, considerable controversy has raged over the separation of the trace isoenzymes of CPK by conventional column chromatographic methods,⁹⁻¹² and quantitation by electrophoretic methods is poor.

The continuous-flow detection system that we are attempting to implement utilizes a coupled sequence of reactions. The enzyme, CPK, catalyzes the conversion of creatine phosphate to creatine, as shown in reaction (1).

Adenosine triphosphate (ATP), a product of the first reaction, phosphorylates glucose in the presence of hexokinase, as shown in reaction (2).

The product, d-glucose-6-phosphate, is dehydrogenated with glucose-6-phosphate-dehydrogenase (GPDH) using NADP as a cofactor, as shown in reaction (3):

The product NADPH is equivalent to the amount of CPK present and is detected by a fluorescence flow monitor.

Only preliminary experimental work has been performed; however, the feasibility of the detection system has been demonstrated. We have been able to separate two of the isoenzymes, CPK-MM and CPK-MB, in ~ 20 min. Control serum samples tested have not contained detectable amounts of CPK-BB. The methodology and instrumentation look promising and are certainly applicable to other isoenzymes separations.

3.2 Blood Sample Preparation System

As stated previously,¹³ genetic mutation is commonly monitored by the determination of variant cellular isoenzymes using electrophoretic techniques coupled with the staining of specific proteins. Statistically meaningful results necessitate the analysis of large numbers of blood samples which, in turn, require a large commitment of technical manpower to prepare the necessary cellular hemolysates for analysis. A system automating procedure of cellular hemolysate preparation has been designed and has the capabilities of: (1) separating whole blood into plasma and cell fractions; (2) washing the cells and collecting portions of plasma and washed cells; and (3) facilitating the preparation of clean hemolysates for electrophoretic analysis.

3.2.1 Background

Studies with previous prototype systems indicated that superior resolution of isoenzymes was achieved by starch gel electrophoresis after the hemolysates obtained from the blood preparation system were subjected to additional centrifugation at 18,000 rpm. These studies also indicated that the addition of a small quantity of Saponin (a commercial hemolyzing

agent) improved lysing. Still further studies, utilizing an improved mixing technique, have shown that lysates exhibiting adequate electrophoretic separation of isoenzymes without additional high-speed centrifugation can be obtained from the system.

3.2.2 Results for a third-generation prototype system

A rotor case of epoxy resin (Epon 828), machined to contain 30 sample chambers, and two processed red-blood-cell collection rings were described in the previous report.¹³ Experimentation with these devices proved disappointing. The epoxy rotor, although it was not subject to attack by lysing solutions, proved to be a very brittle material that was extremely difficult to machine. Once the machining was accomplished, the top and bottom covers had to be attached to the rotor body. Good sealing was only achieved when an epoxy solution of the same formulation as the rotor was used. This solution flowed by capillary action into some of the syphon channels of the rotor, causing them to become plugged. This required additional machining; however, uniform sizes for these syphon channels were never fully realized. Loading of the collection rings required that the operator place each individual cryovial over an "O" ring-sealed protrusion. The difficulty in sealing the cryovial caused blisters on the hands of the operator and was unacceptable. In addition, the drive mechanism did not adequately meet the accelerate/brake requirement to facilitate good mixing. The seal between the drive mechanism and the housing which contains the rotor during the separation process was not sufficient for the containment of liquids (in the housing) which might have resulted from overfilling, leaking, or spillage.

Each of the rotors used in the previous prototype systems has exhibited deficiencies with respect to resuspension of packed cells, especially during the lysing process. The cause is due to several reasons: (1) the accelerate/brake capabilities of the system are inadequate, (2) the lysing solutions react with cell debris (stroma) to form a viscous substance which adheres to the outer wall of the rotor chambers, (3) cell chamber walls are scored by machine tool stress marks which permit stroma to adhere readily to these surfaces, and (4) inadequate optimization of cell chamber size. A rotor designed to simulate blood separation on the Miniature Centrifugal Fast Analyzer was fabricated for the purpose of investigating these problems. This rotor contained four chambers; two were proportional in size and volume to the original prototype system rotor, and the remaining two were proportional in size and volume to the cell chambers contained in the epoxy rotor.

By simulating the blood preparation procedure in a rotor on the portable CFA, we could take advantage of the more rapid accelerate/brake characteristics of the clutch/brake drive mechanisms used in this instrument. It was determined that a rotational rate of 3500 rpm for this rotor in the portable CFA would be equivalent to 2600 rpm for a standard size rotor in the blood preparation system. It was also determined that this rotor, due to size (thickness), could not be safely operated on the portable CFA without modification to the analyzer; therefore, the miniature CFA was used in the simulation studies.

After ensuring that the windows were sealed to the body and that no leaking occurred, the chambers were loaded with proportional (smaller chambers in the simulation rotor) volumes of whole blood, and the simulation was conducted. The results indicated that resuspension of packed

cells occurred, during the accelerate/brake process, in the larger chambers but not in the smaller ones (sizes proportional to chambers used in the epoxy rotor), even after several accelerate/brake steps.

At this point, the rotor was disassembled, and four additional chambers were machined into the rotor body. Two of the new chambers were of an intermediary size compared with the existing chambers, and two were the same size as the smaller chamber but were slightly tapered ($\sim 15^\circ$) at the outer radius. This taper, we felt, would aid in resuspending the cells that had been spun against the chamber wall since they would be moved more easily toward the center of the chamber from an angled, rather than vertical, surface when the rotor was stopped abruptly.

When additional simulation runs were performed with this rotor, it was found that resuspension of cells occurred in the chambers intermediate in size to the initial chambers. It was concluded from the results that chambers proportional to the intermediate size had an optimum radius, and a rotor containing 24 chambers was fabricated from Plexiglass.

3.2.3 Design and fabrication of new prototype system

A fourth-generation prototype blood-sample-preparation system has been designed and fabricated. This system occupies 1 ft^2 of laboratory bench space and stands ~ 18 in. high. An improved mechanical drive mechanisms (illustrated in Fig. 3.2) has been incorporated into this system to provide more rapid acceleration and braking of the rotor. A clutch-brake assembly is used to couple the drive motor to the rotor which provides a much faster and more reliable rotor acceleration than the previous direct-coupled arrangement. This drive mechanisms permits acceleration (from stop) to 2600 rpm in ~ 7 sec as opposed to 12 sec in

Fig. 3.2. Interior view showing mechanical drive mechanism of the improved blood sample preparation system.

the previous system. Braking from 2600 rpm to stop is accomplished in < 1 sec as opposed to 7 sec in the previous system. Thus, better mixing capabilities should result. In addition, the air injection into the rotor chambers appears to be superior (a more even distribution occurs) to the previous system. The unitized clutch-brake assembly should minimize maintenance requirements.

Cleaning of the rotor drive assembly has been simplified by allowing the rotor-holder table to be disassembled from the central drive shaft by removing a single retaining screw. Any spills, leakage, or cleaning solutions are prevented from entering the bearing of the central drive shaft by a spring-loaded shaft seal. This seal also prevents the grease from being forced from the bearing during the pressurization of the rotor during the transfer step.

3.2.4 Sample collection

It was determined that the life of a Plexiglas rotor can be extended dramatically by polishing the machined surfaces that would, in general use, come in contact with organic solutions used in the lysing process. Therefore, the rotor for this system (Fig. 3.3) was fabricated of Plexiglas, and the sample chamber surfaces were polished. The rotor has 24 sample chambers that are sealed from the external environment by the cover seal shown in Fig. 3.3. This seal has an inner and outer O ring and fits around a Rulon® center injection or loading port and is attached to the rotor by three knurled thumb screws. When in place, it enables the proper pressure-to-vacuum ratio to be established, which facilitates the transfer of rotor contents to the collection ring.

The design of the plasma collection ring is essentially unchanged. The top cover extends farther over the chambers than previously, thus

Fig. 3.3. Twenty-four-place rotor with rotary seal and removable "O" ring-sealed cover plate.

restricting access to the collection ports, and will probably necessitate a minor change to enable removal of the samples. The drilling of holes of an appropriate size and angle (from the inner radius toward the outer radius) in this cover to allow plasma removal by a hand-actuated pipet is under consideration.

The most dramatic change in the system has been the method in which hemolysate samples will be collected. A swinging bucket concept is being utilized for this purpose. Figure 3.4 shows an anodized aluminum ring with a stainless steel support rod that fits into a notch-out in the annulus of the ring, to which the cryovial brackets are attached. Figure 3.5 shows the ring with cryovials as it would appear with the rotor in place. As the system is accelerated, the brackets with the cryovials in place change their angle of inclination relative to the rate of rotation. It was experimentally determined that, at a rotational rate of 700 rpm, an angle of inclination of 43° is achieved. This was judged to be sufficient to allow the lysate to be transferred from the rotor into the individual cryovials. Stress calculations were performed for the brackets and bracket mount to ensure that the design was reasonable from a safety viewpoint. These calculations yielded safety factors of 8.2 (tensile stress), 7.5 (tension, 99.9% reliability), and 5.9 (shear) for the mount. Other stress calculations for the ring resulted in equally impressive safety factors.

The wash-solution collection ring has been designed to accomodate the waste solution from three cell washing cycles without removal until cell washing is completed. Figure 3.6 shows this ring in place around the rotor.

Fig. 3 4 Swinging bucket sample ring with cryovials in position.

Fig. 3.5. Twenty-four-place plexiglas rotor assembly with its sample ring containing cryovials in position.

Fig. 3.6. Twenty-four-place plexiglas rotor assembly with wash solution collection ring in place.

A rotor cleaning mechanism consisting of a ring containing 24 stainless steel tubes, drilled to allow a jet stream of water to be directed against the outer radius of the individual sample chamber walls, has been designed. The O rings are used to form a seal, and vacuum aspiration removes cell debris in the wash solution. This mechanism is presently being fabricated. The entire system will be operable in the very near future, and tests will be conducted to determine its effectiveness.

3.3 Automated Elution Electrophoresis*

Development efforts for the automated elution electrophoresis system described earlier¹⁴ have involved investigation of new experimental approaches suggested by a review of the phenomenological theory relevant to the process. These efforts have produced a number of recommendations for future work.

3.3.1 Transport phenomena: current and joule heating

A review of the theory of electrophoresis shows that the phenomenological equations describing the movement of an ion or polyion in an electric field have been developed from analogy to electrostatic problems. The basic problem consisted of describing the force of an electric field on a charged particle in a nonconducting fluid. Inevitable sophistications followed: A counter-ion atmosphere for the charged particle was described; other conducting species in the fluid were considered; and models including interactions between charged particles were developed.

Most descriptions of polyion velocity assumed a uniform electric field throughout the solution. However, separation of a component from the bulk

*The material presented in this section is taken from a comprehensive manuscript, presently in preparation, by W. M. Ayers, a participant in the Exceptional Students Program, summer 1976.

solution during electrophoresis implies a discontinuity of the field at the separated zone-solution interface. The importance of this discontinuity can best be appreciated by examining the transport equations for a solution of charged species between two electrodes. The flux, N_i , of each ionic component can be defined in the absence of any bulk solution movement as:

$$N_i = Z_i C_i U_i \nabla \phi - D_i C_i \quad (1)$$

where

Z_i = charge number of ions;

C_i = molar concentration, moles/cm³;

U_i = species mobility, cm²/volt-sec;

ϕ = field strength, volts/cm;

D_i = diffusion coefficient, cm²/sec.

This assumes that the only electrostatic driving force is that of the external field. Interactions between ion fields of each ion are neglected.

A species balance gives:

$$\frac{dC_i}{dt} = -\nabla N_i \quad (2)$$

The end of the transient period for Eq. (2) signifies the limit of separation for each component. Depending on the initial concentration distribution, field strength, lack of convection, etc., the transient can vary from minutes to hours.

Simplifying to one dimension and assuming that the mobility is constant,

$$N_i = -\left(Z_i C_i U_i \frac{d\phi}{dx} + D_i \frac{dC_i}{dx} \right) \quad (3)$$

and

$$\frac{dC_i}{dt} = U_i \left[\left(\frac{dC_i}{dx} \right) \left(\frac{d\phi}{dx} \right) + C_i \frac{d^2\phi}{dx^2} \right] - D_i \frac{d^2C_i}{dx^2} . \quad (4)$$

The total flux, N , is

$$N = \sum_i N_i , \quad (5)$$

and macroscopic electroneutrality requires

$$\sum_i Z_i C_i = 0 . \quad (6)$$

The current, i , is defined as the total charge that passes through a plane normal to the ion flux,

$$i = FA \sum_i \left[\left(Z_i C_i U_i \right) \frac{d\phi}{dx} - D_i \frac{dC_i}{dx} \right] , \quad (7)$$

where

F = Faraday's constant (96,500 C equiv⁻¹);

A = unit area of normal plane, cm².

Equation (7) illustrates that when a constant current flows between the two electrodes, the local field, $\frac{d\phi}{dx}$, depends on the constituents within the volume or zone of interest. At high ionic strength, the fraction of current carried by the buffer is far greater than that of the polyions being separated. Thus, variations in local field are suppressed by the higher concentration of buffer. The resulting decrease in electrophoretic mobility of polyions with increasing ionic strength in the solution has also been recognized since the early electrostatic formulations. More recent developments in electrophoresis such as discontinuous buffer systems, isotachophoresis, and electrophoresis in water without a buffer take

advantage of this variation in the local field through each zone of polyion to enhance separation.

Another familiar disadvantage of increasing the ionic strength of the solution is the increased convection due to nonuniform joule heating. It can be shown that the joule heat produced in an ionic solution in a cylinder between two electrodes is proportional to the cube of the total ion concentration. The temperature gradient between the axis and the boundary of the cylindrical shell will also follow this cubic dependence on ion concentration as well as its dependence on the square of the tube radius.

3.3.2 Experimental objectives

The experimental objectives suggested by a review of the theory of electrophoresis were:

1. To minimize the consequences of thermal convection by investigating electrophoretic separation in a small diameter tube with low ionic strength solutions, and
2. To investigate the effect of the fluid dielectric constant on polyion mobilities and thus separation times.

In addition, previous experimental work was extended^{13,14} by adding packing to the separation tube and investigating the separation obtained using a high-conductivity (0.01 and 0.10 ionic strength) phosphate buffer.

The sequence of experiments was to investigate protein separation in a small diameter tube filled with water with the electric field applied for successively longer periods until a definite separation as measured photometrically had been achieved. Ideally, this series of experiments would then be repeated, replacing water with fluids having much lower

dielectric constants but not causing protein solubility problems. Finally, packing with phosphate buffers was investigated.

3.3.3 Equipment and materials

The apparatus is shown schematically in Fig. 3.7. The separation tube is attached by a luer fitting on the high-pressure side for easy replacement. Filters (either Whatman No. 2 or a 200-mesh stainless steel screen for high- and low-conductivity experiments, respectively) were used to contain the packing during elution. The separation tube was 10 cm long by 0.1-cm-ID quartz or Teflon (Penntube AWG-18-TW, TFE).

Packing when used was Corning CPG glycophas G, 75 to 125- μ particles with 100 Å pores. A hydrophilic coating (18 Å thick) covered the exterior and pore surfaces of the particles; this coating was stable over the pH range of 0 to 9 and in a number of common solvents.

Supporting fluids or solvents for the experiments were distilled water and distilled water plus dibasic sodium phosphate (Na_2HPO_4) at 0.01 and 0.1 ionic strengths. Water was chosen for the high-field experiments (5 to 10 kV) since it has a low dissociation constant and, therefore, low conductivity.

Three protein mixtures were used to test the resolution of the system. These were albumin/gamma globulin (A- γ -1), Versatol (human serum), and a goat serum with IgM antiserum (G-S-IgM). Analysis of ions for all mixtures are presented in Table 3.3.

Since the temperature distribution in the unpacked tube should be proportional to the square of the tube radius and cube of total ion concentration, both of these variables should be minimized. However, the total ion concentration in water is greatly increased by ions in the

Fig. 3.7. Schematic of apparatus to study the effect of separations media on electrophoretic transport.

protein mixtures (see Table 3.3). The tube radius was limited by available materials and a desire to minimize sample injection errors.

Additional equipment included a Hiptronics power supply (0 to 5 kV, 0 to 200 mA), a Dumont power supply (0 to 12 kV, 0 to 7 mA), and a UV flow monitor.

Table 3.3. Protein and ion constituents of sample mixtures

	Versotol	A-γ-1	G-S-IgM
<u>Proteins, (g/100 ml)</u>			
Albumin	4.1	3.2	a
Globulins			
α-1	0.3		
α-2	0.9	0.8	
β	0.6		
γ	1.2		
IgM			0.045
<u>Ions (mg/ml)</u>			
	b	c	c
Ca ²⁺	0.102	12.0	84.0
Mg ²⁺	0.022	0.49	25.2
K ⁺	1.955	6.00	153
Na ⁺	32.18	39.0	3660
Cl ⁻	36.52		

^aNot available from manufacturer; Hoechst Pharmaceuticals, Somerville, New Jersey.

^bManufacturer's analysis; General Diagnostics, Morris Plains, New Jersey.

^cAnalysis by Environmental Sciences Division, ORNL.

3.3.4 Experimental procedure

Once the separation tube, packing, and solvent have been chosen, the experiment begins with the following sequence:

1. Flush the tube with several column volumes of eluent to remove trapped air.
2. Fill and balance levels of solvent in both electrode chambers; turn off eluent.
3. Set ampere meter to the range expected for maximum current flow.
4. Set attenuation on recorder for current range and sample concentration deflections.
5. Inject a 2- μ l sample; close valve.
6. Check that both three-way valves are open for flow between electrode chambers.
7. Turn on power supply, adjust voltage to predetermined level.

After the electrophoresis period:

1. Set the three-way valve for column elution.
2. Empty anode (+) chamber to prevent backflow through detector.
3. Increase recorder chart speed.
4. Turn on eluent pump at predetermined flow rate.

After all traces of sample have been removed from the separation tube, empty and replace solvent in both electrode chambers.

The maximum voltage applied to the apparatus is determined with only solvent (and packing) in the tube. The voltage is increased incrementally until the current through the tube drops to zero. The tube is then flushed, and voltage is increased to slightly less than the previous breakdown voltage. The objective is to obtain a nearly constant current at maximum

voltage for the duration of the experiment. In practice, the current is dependent on the duration of the applied potential. The duration of the applied field ranged from 10 min to 15 hr.

The apparatus is designed so that a protein, during electrophoresis, can pass through the uv detector. If this does not occur, the sample can be eluted from the column.

A Buchler Polystaltic or Beckman AccuFlo pumped eluent (the same solvent used in the electrode chambers) through the tube from a reservoir.

3.3.5 Results and discussion

Representative experimental conditions (from ~ 70 experiments) are given in Table 3.4. In runs 3 to 5, only the sample size was varied; marginal separation between (apparently) albumin and globulins was achieved but not reproducibly. The variation of current with time, even for this simple system, showed no discernible regularity other than an increase with sample size.

In runs 6 to 11, packing was added to the system. In these runs, no peaks occurred during the field application, but marginal separation was again obtained between (apparently) albumin and globulins. Essentially the same results were obtained in runs 12 to 16; however, obviously longer run times were required to achieve these results.

The reason for the absence of reproducible results in these experiments is not clear. However, the inability to duplicate the field through the separation tube, as indicated by monitoring of the current, suggests that the conductivity through the packing varies with each experiment. It should be noted that the instability of the current, in the packed tube, increases with the ionic strength of the solution.

Table 3.4 Representative experimental conditions for experiments

Fluid	Supply voltage	Duration	Eluent (ml/sec)	Run No.	
<u>No packing (quartz tube)</u>					
A- γ -1 (2 μ l)	Water	5 kV	30 min	0.008	3
A- γ -1 (3 μ l)	Water	5 kV	28 min	0.007	4
A- γ -1 (1 μ l)	Water	5 kV	20 min	0.005	5
<u>With Packing</u>					
G-S-IgM (1 μ l)	Water	5 kV	45 min	0.052	6
G-S-IgM (1 μ l)	Water	5 kV	15 min	0.015	7
G-S-IgM (2 μ l)	Water	5 kV	30 min	0.015	8
G-S-IgM (2 μ l)	Water	5 kV	40 min	0.015	9
G-S-IgM (2 μ l)	Water	5 kV	50 min	0.011	10
G-S-IgM (2 μ l)	Water	5 kV	61 min	0.015	11
G-S-IgM (2 μ l)	Water ($\Gamma = 0.01$) ^a	2 kV	16 hr	0.016	12
G-S-IgM (2 μ l)	Water ($\Gamma = 0.1$)	1 kV	15 hr	0.016	13
G-S-IgM (2 μ l)	Water ($\Gamma = 0.1$)	1 kV	7.5 hr	0.004	14
G-S-IgM (2 μ l)	Water ($\Gamma = 0.1$)	500 V	15.5 hr	0.004	15
Versotol (2 μ l)	Water ($\Gamma = 0.1$)	900 V	15 hr	0.016	16

^a Γ = ionic strength.

3.3.6 Recommendations

If elution electrophoresis is to be developed in a continuous buffer system with convection suppressed by particulate packing, the packing should have interparticle voids of approximately the same magnitude as the mean diameter of the proteins. If resolution comparable to acrylamide gels is desired, the experimental apparatus should be equipped with high-pressure fittings.

Since conductivity of the protein solutions is a function of time, a constant current (rather than constant voltage) power supply should be obtained. Reproducible separations will not be achieved unless the operating characteristics (i.e., the current through the potential across the separating tube) are reproducible. Resolution might be improved by the addition of surfactants to the protein mixture.

If packing can be omitted, further improvements in macromolecular separation are possible. Ideally, such improvements will require an apparatus in which the transport equations and boundary conditions for the separation zone are well defined. The traditional problem with high-conductivity fluids has been convection due to temperature gradients. The historical approach has been to suppress this convection with packing or cooling at the boundaries. More recently, it has been realized that if the bulk fluid has a momentum, thermally-induced convection (or other instabilities) must, to be significant, have sufficient energy to perturb this momentum. Thus, imparting sufficient motion to the entire fluid eliminates random instabilities and allows the electrophoretic transport to be modeled in a defined environment. Kolin¹⁵ devised a clever device to electromagnetically impart a bulk rotational flow to a solution during

electrophoresis. Another variation of this concept involved mechanically rotating the outer cylinder of the annulus to rotate the fluid.

In general, the investigation of the stated objectives has been preliminary in nature. It is recommended that future development work on automated elution electrophoresis include further investigation of the methods suggested by the review of the theory of electrophoresis and its current practice.

3.4 References for Section 3

1. C. D. Scott et al., Experimental Engineering Sect. Semiannu. Prog. Rep. (Excluding Reactor Programs), Sept. 1, 1974 to Feb. 28, 1975, ORNL/TM-4961 (1975).
2. J. E. Mrochek et al., Biochemical Technology Program Prog. Rep. for the Period January 1-June 30, 1976, ORNL/TM-5446 (1976).
3. A. Kolin and S. J. Luner, "Endless Belt Electrophoresis," in Progress in Separation and Purification, vol. 4, ed. by E. S. Perry and C. J. Van Oss, Wiley-Interscience, New York, 1971.
4. R. J. Kudirka, R. R. Schroeder, T. E. Hewitt, and E. C. Toven, Jr., "High-Pressure Liquid Chromatographic Separation of Lactate Dehydrogenase Isoenzymes," Clin. Chem. 22, 471 (1976).
5. M. A. Varat and D. W. Mercer, "Cardiac Specific Creatine Phosphokinase Isoenzyme in the Diagnosis of Acute Myocardial Infarction," Circulation, 51(5), 855 (1975).
6. R. S. Galen, J. A. Rieffel, and S. R. Gambino, "Diagnosis of Acute Myocardial Infarction," J. Am. Med. Assoc. 232, 145 (1975).
7. P. L. Wolf, T. Kerns, J. Neuhoﬀ, and J. Lauridson, "Identification of CPK Isoenzyme MB in Myocardial Infarction," Lab. Med. 5(7), 48 (1974).
8. G. S. Wagner, C. R. Roe, L. E. Limbird, R. A. Rosati, and A. G. Wallace, "The Importance of the Identification of the Myocardial-Specific Isoenzyme of Creatine Phosphokinase (MB Form) in the Diagnosis of Acute Myocardial Infarction," Circulation 47, 263 (1973).
9. P. C.-P. Wong and A. F. Smith, "Comparison of 3 Methods of Analysis of the MB Isoenzyme of Creatine Kinase in Serum," Clin. Chem. Acta. 65, 99 (1975).
10. G. Lum and A. L. Levy, "Chromatographic and Electrophoretic Separation of Creatine Kinase Isoenzymes Compared," Clin. Chem. 21, 1601 (1975).

11. W. G. Yasmineh and N. Q. Hanson, "Electrophoresis on Cellulose Acetate and Chromatography on DEAE-Sephadex A-50 Compared in the Estimation of Creatine Kinase Isoenzymes," Clin. Chem. 21, 381 (1975).
12. S. M. Sax, J. J. Moore, J. L. Giegel, and M. Welsh, "Atypical Increase in Serum Creatine Kinase Activity in Hospital Patients," Clin. Chem. 22, 87 (1976).
13. J. E. Mrochek, C. A. Burtis, and C. D. Scott, Biochemical Technology Program Prog. Rep. for the Period January 1-June 30, 1976, ORNL/TM-5446 (September 1976).
14. C. D. Scott et al., Experimental Engineering Sect. Semiannu. Prog. Rep. (Excluding Reactor Programs), Sept. 1, 1974 to Feb. 28, 1975, ORNL/TM-4961 (1975).
15. A. Kolin and S. J. Luner, "Endless Belt Electrophoresis," in Progress in Separation and Purification, vol. 4, ed. by E. S. Perry and C. J. Van Oss, Wiley-Interscience, New York, 1971.

4. BIOENGINEERING RESEARCH

B. Z. Egan, W. W. Pitt, Jr., and S. E. Shumate II

Bioengineering research has been conducted in two areas during this report period: (1) enzyme catalysis and (2) tritium isolation by bacteria.

Studies were continued on: (1) purification and separation of the ferredoxin and hydrogenase enzymes, (2) a search for organic solvents capable of dissolving the immobilizing enzymes, and (3) studies related to the rate of oxidation of ferredoxin. A multiple-step procedure involving precipitation, heat treatment, DEAE-cellulose chromatography, and gel permeation chromatography was studied for partial purification of ferredoxin and hydrogenase. Although enzymatic activity was retained throughout the procedure, significant separation of the hydrogenase and ferredoxin was not achieved. Over 25 organic solvents and organic solutions were studied as possible extractants or solvents for enzymes, including amines, carboxylic acids, an alkylated phenol, and an alkyl phosphoric acid. Although in many cases, the protein (lactase) was depleted in the aqueous phase, an emulsion or precipitate frequently formed, and the transfer to the organic phase was not confirmed. Methods are being developed for measuring the rates of oxidation and reduction of ferredoxin.

At the request of ERDA, a scouting study was begun in July, 1976, to investigate a microbiological hydrogen-isotope separation phenomenon which has been reported for deuterium and to determine whether a similar effect could be detected for tritium. Such an effect could possibly be utilized as a means of tritium isolation and removal from fuel-reprocessing effluent streams. In such an application, tritiated water would be concentrated with respect to tritium so that the volume of tritium-enriched

water to be subsequently stored or otherwise disposed of is reduced drastically. Experimental procedures and apparatus to study cell growth and hydrogen evolution were established. Parallel experiments are being conducted. One is an "approach-to-equilibrium" technique wherein gas resulting from the fermentation will be collected by the displacement of the medium from the incubation flask. The second and more interesting experiment (from a process application viewpoint) utilizes a nonequilibrium technique. As the gas is evolved in the incubation flask, it is removed by displacement of mercury from a separate vessel, thus eliminating the possibility of HT isotope exchange with the displaced fluid. During these studies, the emphasis will be to determine rates of tritium concentration, if any, as a function of species (e.g., strain G4A) and operating parameters (e.g., carbon substrate, additional nutrients, cell concentration, tritium concentration, temperature, dissolved O_2 and CO_2 , mixing rate).

4.1 Enzyme Catalysis

B. Z. Egan and J. P. Eubanks

Studies are continuing¹⁻³ on the enzyme-catalyzed reduction of water with sodium dithionite for the production of hydrogen. During this report period, efforts were concentrated in three areas: (1) purification and separation of the ferredoxin and hydrogenase enzymes, (2) a search for organic solvents capable of dissolving and immobilizing enzymes, and (3) studies related to the rate of oxidation of ferredoxin.

4.1.1 Purification and separation of ferredoxin and hydrogenase

Much of our previous work has utilized whole cell extracts containing ferredoxin and hydrogenase or ferredoxin obtained from a commercial source (Sigma Chemical Company). In enzyme immobilization studies, higher enzyme activity and better efficiency can be obtained by using purified preparations from which extraneous, and possibly interfering, proteins have been removed. Purified preparations of ferredoxin and hydrogenase are also necessary for measuring oxidation rates of the individual components.

Partial purification and separation of ferredoxin and hydrogenase have been reported.⁴⁻⁸ Multiple steps involving precipitation, heat treatment, DEAE-cellulose chromatography, and gel permeation chromatography were required. Because of its high affinity for DEAE-cellulose, ferredoxin can be prepared in reasonable purity and can be stored for several weeks. Purification of hydrogenase is more difficult. Procedures are complicated by the necessity of excluding air (oxygen) from the solutions to prevent oxidation.

Cell extracts containing hydrogenase and ferredoxin were obtained from *Clostridium pasteurianum* as previously described.¹⁻³ The protein content was detected and monitored by the absorbance of the solutions at 280 nm. In earlier chromatographic runs, fractions were collected and measured individually. A glove box has now been equipped with a continuous flow, variable wavelength monitor for monitoring column effluents.

Columns of DEAE-cellulose and Sephadex G-100 were prepared in 10- and 50-ml syringes equipped with stopcocks were assembled in the glove box. A two-chambered vessel was used to generate a linear salt gradient for eluting the samples when required.

Enzyme activity of various samples and chromatographic fractions was measured by the rate of hydrogen formation upon reaction with sodium dithionite substrate. The activity of recombined fractions was compared with the activity of the separate fractions to determine whether separation was achieved.

A procedure for separation of ferredoxin and hydrogenase was adapted and developed in which the following steps were used. Protamine sulfate was added to the cell extract to precipitate nucleic acids. The resulting supernatant was heated to 50°C for 10 to 15 min, then cooled to 0°C for 10 to 15 min. The resulting precipitate was removed by centrifugation and discarded. The supernatant was chromatographed on a DEAE-cellulose column (Cl⁻ form) using a potassium chloride (KCl) gradient in Tris-Cl buffer. Protein-containing fractions from the DEAE-cellulose column were assayed for enzyme activity, and selected fractions were chromatographed on a Sephadex G-100 column. Selected fractions from the G-100 column were assayed for enzyme activity using sodium dithionite substrate.

Protein recovery using the above procedure is summarized in Table 4.1 for a typical sample. Chromatography on DEAE-cellulose (step 4) using a KCl gradient gave a single peak. When the fractions containing material represented by this peak were combined and chromatographed on a G-100 column, two peaks were obtained. When assayed for enzyme activity, the first peak alone produced hydrogen; however, the second peak did not. Also, the addition of the second peak to the first peak did not increase the rate of hydrogen production.

Table 4.1. Protein recovery from enzyme purification steps

Step	Solution volume (ml)	Absorbance (280 nm)	Total protein (A_{280} units)	Recovery (%)
1. Cell extract	5.7	102	580	100
2. Supernatant from protamine sulfate	5.7	98	560	97
3. Supernatant from heat treatment	4.1	70	290	50
4. Fractions from DEAE-cellulose	31		220	38
5. Fractions from G-100	80		200	34

A larger amount of sample was carried through a similar procedure, and the enzyme activity was determined after the heat treatment, DEAE-cellulose chromatography, and G-100 chromatography. Fifteen milligrams of protamine sulfate was mixed with about 11 ml of cell extract. The recovered supernatant was heated at 50°C for 15 min and then cooled in an ice bath for 15 min. The resulting supernatant solution contained $\sim 120 A_{280}$ units/ml. The enzyme activity of the heat-treated extract was compared with the activity of the original cell extract, using 0.5 ml of the original cell extract and 0.6 ml of the heat-treated extract to obtain equal amounts of protein (A_{280} units). As shown in Fig. 4.1, there was no significant change in activity as a result of the heat treatment.

The heat-treated sample (~ 8 ml containing 790 A_{280} units) was chromatographed on a DEAE-cellulose column using a 40-ml linear gradient of 0 to 0.3 M KCl in 0.05 M Tris-Cl (pH 7.0) followed by 0.3 M KCl, and

ORNL DWG. 76-14719

Fig. 4.1. Comparison of ferredoxin-hydrogenase activity of cell extract (curve 1) and cell extract after protamine sulfate and heat treatment (curve 2). Solutions contained 35 mg of sodium dithionite in 10 ml of 0.05 M potassium phosphate buffer (pH 6.6) and equivalent amounts (A_{280} units of protein).

finally 0.6 M KCl. A large peak, peak 1 ($\sim 470 A_{280}$ units), eluted near 0.1 M KCl. A smaller peak, peak 2 ($\sim 70 A_{280}$ units), eluted near 0.3 M KCl. Finally, peak 3, containing $\sim 190 A_{280}$ units, was eluted with 0.6 M KCl. Both a dark brown band and a yellow band were visible on the column. The dark band was eluted with the 0.3 M KCl, and the yellow band was eluted with the 0.6 M KCl. Peaks 1 and 3 were assayed for enzyme activity, whereas peak 2 did not contain sufficient protein for the assay. Peak 1 produced hydrogen; peak 3 did not. Recombination of peak 3 with peak 1 did not significantly improve the hydrogen production of peak 1.

Approximately 250 A_{280} units of peak 1 from the DEAE-cellulose column was chromatographed on a G-100 column. Two peaks were obtained: a minor peak, $\sim 25 A_{280}$ units, eluted near the void volume, and a major, broad peak, $\sim 200 A_{280}$ units, eluted near a column volume. When assayed for enzyme activity, the first peak produced hydrogen; however, the second peak did not.

We are continuing efforts to improve the enzyme purification and separation procedures. One of the obvious problems with the procedures described is the successive dilution of the sample during chromatography, resulting in large volumes of dilute enzymes. We have found that ultrafiltration is a successful method of concentrating the enzyme solutions and can be done in a glove box. An ultrafilter supported on a hollow cylinder (Immersible Molecular Separators, Millipore Corporation) was immersed in cell extract solution, and the inside of the cylinder was connected to an evacuated tube (Vacutainer). The solution volume was reduced from 4.8 to 2.2 ml in 2 hr. As shown in Fig. 4.2, there was no loss in enzyme activity. In fact, there was a slight enhancement of the initial hydrogen production rate over that expected from the concentration

ORNL DWG. 76-14720

Fig. 4.2. Comparison of ferredoxin-hydrogenase activity of cell extract (curve 1) and retentate (curve 2) from Millipore ultrafilter. Solutions contained 35 mg of sodium dithionite in 10 ml of 0.05 M potassium phosphate buffer (pH 6.6) and 0.5 ml of enzyme solution.

factor alone. This ultrafiltration method should be equally effective for concentrating chromatographic fractions.

4.1.2 Enzyme stabilization and immobilization

The most common methods of enzyme immobilization involve the use of a solid matrix. In this method, the enzyme is attached by adsorption, entrapment, or covalent binding. We previously reported⁹ the adsorption of the ferredoxin-hydrogenase system as well as lactase on RPC-5. The RPC-5 material consists of polychlorotrifluoroethylene powder coated with Adogen 464, a quaternary ammonium chloride. Also, in one experiment a ferredoxin solution containing 6 mg of ferredoxin in 3 ml of phosphate buffer was added to 4 g of RPC-5. After mixing for 30 min at room temperature, the ferredoxin was completely removed from the solution, as measured by the absorbance at 280 nm. Accordingly, we postulated that perhaps enzymes could be immobilized by solubilization in organic solvents. If organic solvents that are immiscible with and insoluble in aqueous solutions could be found, then reactions between substrates in the aqueous phase and enzymes in the organic phase could be carried out in a conventional two-phase contactor in which the enzyme was retained in the organic phase and recycled.

There is very little information on the behavior of enzymes in organic solutions. A search was begun for organic solvents or solutions that would extract enzymes from aqueous solutions. In preliminary tests thus far, aqueous solutions of lactase were used. Lactase is inexpensive and readily available, and assay procedures are well defined. The aqueous phase contained 0.2 M NaH_2PO_4 , 0.1 M citric acid (pH 3.5), and 5 to 10 mg of lactase per milliliter. The amount of enzyme in the aqueous phase was measured by the absorbance at 280 nm. The absorbance of the aqueous phase

was measured before (feed) and after (raffinate) mixing with the organic phase. Some of the various organic solvents and solutions of potential enzyme extractants that were tested are listed in Table 4.2. Organic phases containing an extractant were 0.2 M in extractant concentration. Equal volumes of each phase were mixed at room temperature. Extractants included amines, organic acids, and a substituted phenol. Also, mixtures of polyethylene glycol and dextran polymers that form two phases in aqueous solution¹⁰ were used.

In many cases, the concentration of lactase in the aqueous phase decreased on contact with the organic. In some cases, the simple solvents were more effective than the organic solutions. However, an emulsion or crud frequently formed at the interface, and the transfer of the enzyme to the organic phase was not confirmed by direct measurement in the organic phase. Some of the enzyme may have been precipitated at the interface. We are continuing the search for useful, aqueous, immiscible solvents and will investigate the more promising ones for use with the ferredoxin-hydrogenase system.

4.1.3 Rate of oxidation of ferredoxin

Several proposed schemes for energy production utilize ferredoxin as an electron carrier. In most schemes, the oxidation of ferredoxin by oxygen is detrimental, and oxygen must be excluded from the system. In some schemes, molecular oxygen is produced in the presence of the reduced ferredoxin. There are conflicting reports concerning the stability of ferredoxin in the presence of oxygen, and quantitative data are lacking on the rate of oxidation of ferredoxin with molecular oxygen. An objective of our program is to measure the rate of oxidation of ferredoxin.

Table 4.2. Solvent extraction behavior of lactase

Extractant	Diluent	Aqueous feed (A ₂₈₀)	Aqueous raffinate (A ₂₈₀)
	Chloroform	1.70	0.30
	Trichloroethylene	1.70	0.32
	1,2-dichloroethane	1.70	0.39
	Tetrachlorotetra- fluoropropane	1.70	0.51
	Dodecane	1.70	0.92
	Cyclohexane	1.70	0.62
1-nonyldecylamine	Chloroform	1.64	1.21
1-nonyldecylamine	Dodecane	1.64	1.21
1-nonyldecylamine	1,2-dichloroethane	1.23	0.69
Amberlite XLA-3	Chloroform	1.64	1.25
Amberlite XLA-3	Dodecane	1.64	1.29
Amberlite XLA-3	1,2-dichloroethane	1.23	0.78
3,9-diethyltridecyl-6-amine	Chloroform	1.64	1.29
3,9-diethyltridecyl-6-amine	Dodecane	1.64	1.29
3,9-diethyltridecyl-6-amine	1,2-dichloroethane	1.23	0.72
Amberlite LA-1	Chloroform	1.68	1.31
Amberlite LA-1	Dodecane	1.68	1.24
Alamine 336	Chloroform	1.68	1.21
Alamine 336	Dodecane	1.68	1.38
Adogen 464	Chloroform	1.64	0.97
Adogen 464	Dodecane	1.68	1.32
4- <u>sec</u> -butyl-2(α -methylbenzyl)phenol	Chloroform	1.72	1.02
4- <u>sec</u> -butyl-2(α -methylbenzyl)phenol	Dodecane	1.68	1.60
4- <u>sec</u> -butyl-2(α -methylbenzyl)phenol	1,2-dichloroethane	0.82	0.54
Di-2-ethylhexylphosphoric acid	Chloroform	1.70	1.31
Naphtenic acid	1,2-dichloroethane	0.82	0.52
5-phenylvaleric acid	1,2-dichloroethane	0.82	0.59
Neotridecanoic acid	1,2-dichloroethane	0.82	0.61

One approach is to measure the depletion of oxygen in solution with ferredoxin using an oxygen-sensing electrode. Such electrodes are commercially available and have been used successfully for monitoring the oxygen content of a variety of aqueous solutions.

There is also a significant difference in the uv-visible spectra of oxidized and reduced ferredoxin.^{11,12} Oxidized ferredoxin has a significantly higher absorbance than the reduced form at 390 to 400 nm. This difference can be utilized for analytical purposes.

It is first necessary to ensure that the ferredoxin is completely reduced (or the amount in the reduced form is known) before measuring the rate of oxidation of ferredoxin. Ferredoxin can be reduced with sodium dithionite. However, any excess dithionite will react with oxygen, as will the ferredoxin. Consequently, the use of dithionite as a reducing agent required that the rate of dithionite oxidation be either much faster or slower than the rate of oxidation of ferredoxin so that the separate rates are distinguishable. Otherwise, stoichiometric amounts of dithionite must be used. The stoichiometry is not well defined.

Preliminary experiments indicate that the rate of oxidation of dithionite is relatively fast. A buffer solution containing 0.14 mg of sodium dithionite per milliliter reduced the oxygen content of the solution from 8.6 to 0.4 ppm in 30 sec, as indicated by the oxygen-sensing electrode. In another solution containing 0.029 mg of dithionite per milliliter, the oxygen concentration was decreased from 6.4 to 2.2 ppm in < 1 min. We are presently trying to determine whether the rate of oxidation of ferredoxin is faster or slower than the rate of oxidation of dithionite. Thus far, the oxygen electrode has not been sufficiently

stable to measure rates that require continuous monitoring for several hours. The electrode slowly drifts toward lower oxygen values when standing in buffer solution. For example, a solution indicating 9.1 ppm oxygen decreased to a value of 5.6 ppm in 16.5 hr.

Results obtained thus far with mixtures of sodium dithionite and ferredoxin are inconclusive. We are also considering the possible use of the Centrifugal Fast Analyzer (CFA) for determining the rate of oxidation of ferredoxin by measuring the absorbance change at 400 nm.

4.2 Tritium Isolation by Bacteria

At the request of ERDA, a scouting study was begun in July, 1976, to investigate a microbiological hydrogen-isotope separation phenomenon which has been reported for deuterium¹³⁻¹⁵ and to determine whether a similar effect could be detected for tritium. Such an effect could possibly be utilized as a means of tritium isolation and removal from fuel reprocessing effluent streams. In such an application, tritiated water would be concentrated with respect to tritium so that the volume of tritium-enriched water to be subsequently stored or otherwise disposed of is reduced drastically.

Work thus far has focused on development of experimental procedures and apparatus to study cell growth and hydrogen evolution. The work of Krichevsky et al.¹⁵ has been used as a foundation for the development of a detailed experimental protocol. Experiments are being conducted along two lines. The first is an approach-to-equilibrium technique wherein gas resulting from the fermentation will be collected by displacement of the medium from the incubation flask. Thus, the gas will remain in contact with the medium, through the gas-liquid interface, during the

incubation period of ~ 48 hr. By carefully controlling our experimental conditions, we expect to approach an equilibrium for the hydrogenase-catalyzed isotope exchange reaction:

Analysis of the gas and liquid phases by a combination of gas chromatography, ionization chamber counting, and liquid scintillation counting will permit computation of an equilibrium constant for the exchange reaction. Comparison with literature values¹⁶⁻¹⁷ will give us an indication of our experimental accuracy.

The second, and more interesting (from a process application viewpoint), type of experiment utilizes a nonequilibrium technique. Such experiments will be performed under incubation conditions identical to those for the approach-to-equilibrium case. However, the gas will be removed from the incubation flask as it is evolved. This will be accomplished by the displacement of mercury from a separate vessel. Herein, we will have eliminated the possibility of HT isotope exchange with the displaced fluid - a noteworthy departure from the Krichevsky technique. At the end of the incubation period, the bacterial cells (strain G4A) will be recovered by centrifugation. Subsequently, they will be digested in a tissue solubilizer compatible with the liquid scintillation fluid and counted from tritium. Analysis of the gas and liquid (cell-free) phases will be conducted as previously described.

On the basis of Krichevsky's results for deuterium, we hope to measure a depletion of tritium in the evolved gas (less than the equilibrium concentration) and to determine disposition of tritium within the

bacterial cell. A check will be made for the possibility of isotope exchange with medium constituents. After ~ 48 hr, water ($H_2O + HTO$) will be evaporated from the cell-free medium, condensed, and recounted for tritium. Apparently, Krichevsky did not make such a check. During these studies, the emphasis will be on determining rates of tritium concentration, if any, as a function of species (e.g., strain G4A) and operating parameters (e.g., carbon substrate, additional nutrients, cell concentration, tritium concentration, temperature, dissolved O_2 and CO_2 , mixing rate). Results will be reported in future progress reports.

4.3 References for Section 4

1. C. D. Scott et al., Experimental Engineering Section Semiannu. Prog. Rep. (Excluding Reactor Programs) Mar. 1, 1975 to Aug. 31, 1975, ORNL/TM-5291 (September 1976).
2. C. D. Scott et al., Experimental Engineering Section Semiannu. Prog. Rep. (Excluding Reactor Programs) Sept. 1, 1974 to Feb. 29, 1975, ORNL/TM-4961 (January 1976).
3. C. D. Scott et al., Experimental Engineering Section Semiannu. Prog. Rep. (Excluding Reactor Programs) Mar. 1, 1974 to Aug. 31, 1974, ORNL/TM-4777 (July 1975).
4. L. E. Mortenson and J-S. Chen, p. 256 in Microbial Iron Metabolism - A Comprehensive Treatise, ed. by J. B. Neilands, Academic Press, New York, 1974.
5. N. A. Stombaugh, R. H. Burris, and W. H. Orme-Johnson, "Ferredoxins from Bacillus polymyxa," J. Biol. Chem. 248, 7951 (1973).
6. G. Nakos and L. Mortenson, "Purification and Properties of Hydrogenase, an Iron Sulfur Protein, from Clostridium pasteurianum W5," Biochim. Biophys. Acta. 227, 576 (1971).
7. B. B. Buchanan, W. Lovenberg, and J. C. Rabinowitz, "A Comparison of Clostridial Ferredoxins," Proc. Nat. Acad. Sci. 49, 345 (1963).
8. R. C. Valentine, L. E. Mortenson, and J. E. Carnahan, "The Hydrogenase System of Clostridium pasteurianum," J. Biol. Chem. 238, 1141 (1963).
9. C. D. Scott et al., Experimental Engineering Section Semiannu. Prog. Rep. (Excluding Reactor Programs) Sept. 1, 1975 to Feb. 29, 1976, ORNL/TM-5533 (in preparation).

10. R. Fleischaker and D. I. C. Wang, 172nd National American Chemical Society Meeting, San Francisco, California, Aug. 29-Sept. 3, 1976.
11. K. Uyeda and J. C. Rabinowitz, "Pyruvate-Ferredoxin Oxidoreductase," J. Biol. Chem. 246, 3111 (1971).
12. R. Malkin and J. C. Rabinowitz, "Additional Observations on the Chemistry of Clostridial Ferredoxin," Biochemistry 5, 1262 (1966).
13. P. E. Cloud, I. Friedman, F. D. Sisler, and V. Diebler, "Microbiological Fractionation of the Hydrogen Isotopes," Science 127, 1394 (1958).
14. F. D. Sisler, "Biogeochemical Concentration of Deuterium in the Marine Environment," Science 129, 1288 (1959).
15. M. I. Krichevsky, I. Friedman, M. F. Newell, and F. D. Sisler, "Deuterium Fractionation During Molecular Hydrogen Formation in a Marine Pseudomonad," J. Biol. Chem. 236, 2520 (1961).
16. D. G. Jacobs, "Sources of Tritium and Its Behavior Upon Release to the Environment," TID-24635, U.S. Atomic Energy Commission (1968).
17. S. R. Anand and A. I. Krasna, "Catalysis of the H₂-HTO Exchange by Hydrogenase. A New Assay for Hydrogenase," Biochemistry 4, 2747 (1965).

5. BIOENGINEERING DEVELOPMENT

A. L. Compere, W. L. Griffith, C. W. Hancher, D. D. Lee
D. L. Million and W. W. Pitt, Jr.

During this report period, development efforts in bioengineering have been concentrated in four areas: (1) development of the ANFLOW Process for treatment of municipal wastewaters, (2) development of a bioprocess for the treatment of coal conversion aqueous effluents, (3) support of the nitrate waste recycle facility at Y-12, and (4) tapered fluidized-bed bioreactor studies.

Two areas of vital national concern, energy conservation and water pollution, converge in the treatment of aqueous wastes, since conventional aerobic waste water treatment methods are energy-intensive. Fuel production and energy conservation are potentially possible in wastewater treatment facilities utilizing a new bioreactor, the ANFLOW bioreactor. The objective of this program is to evaluate the ANFLOW bioreactor for the treatment of domestic and industrial wastewaters and to investigate its potential by determining the system operating parameters, including energy requirements, fuel gas production, capital investment, and the quality of waste treatment afforded. During this report period, an ANFLOW Demonstration Pilot Plant has been designed and construction has been initiated at the Oak Ridge East Waste Treatment Plant.

Phenols, thiocyanates, and ammonia are the major contaminants to be removed from coal-conversion aqueous waste streams. The biological degradation of phenol and thiocyanate in a three-phase, tapered fluidized-bed bioreactor (TFBBR) is being studied. During this report period, the degradation of thiocyanate using either a commercially available culture, Phenobac[®], or an activated sludge from Bethlehem Steel has been investigated.

The biotechnology development staff in the Advanced Technology Section (ATS) has maintained a continuing interest and effort in biodenitrification as well as periodic interaction with the staff of the Y-12 Development Division, which is responsible for the design, installation, and start-up of the Nitrate Waste Recycle Facility at Y-12. It was agreed that the ATS biotechnology staff assist the Y-12 biodenitrification group with several specific tasks: (1) backup storage of seed bacteria, (2) bacterial growth during reactor start-up, and (3) determinations of uranium and plutonium balance in bioreactor sludge. Seed bacteria are kept frozen and stored in two freezers, and samples are periodically removed to check for viability. From the results it was concluded that sudden pH changes and low carbon-to-nitrogen ratios in the feed should be avoided, particularly during start-up. Two of the tests were continued to determine the effects of high levels of chromium and uranium on bacterial growth and bioreactor operation. Results showed that concentrations of chromium up to 400 $\mu\text{g/ml}$ or uranium up to 500 $\mu\text{g/ml}$ apparently had no adverse effect on denitrification rates of bacterial population. During these tests, analyses for uranium and chromium showed that > 97% of dissolved chromium and > 60% of dissolved uranium in the feed are removed by the sludge and free cells.

Efforts are continuing to determine the wide range of operating capabilities and characteristics of the TFBRR. During this report period, two different tests were made - one to demonstrate TFBRR utility in the production of a valued product and the other to elucidate the effects of varying the taper angle. The TFBRR was used as a continuous fermentor

for the conversion of glucose to ethanol. Initial results indicated that a longer residence time was required; hence, a 13-ft high TFBRR, consisting of a 9-ft straight section, a 2-ft tapered section, and finally a 2-ft straight section was constructed. Successful operation of this bioreactor demonstrated that this is a viable method of increasing residence time. A variable angle test unit was constructed to determine the effect of taper angle on hydraulic and mass transfer performance of tapered fluidized beds. During this report period, the hydraulic pressure drop, expanded bed height, and void volume as a function of fluid flow rate were determined for five different taper angles with a 500-g bed of coal.

5.1 ANFLOW Bioreactor

Energy conservation is an increasingly important factor in achieving domestic energy self-sufficiency. Energy-saving technologies must be developed and applied on a large scale throughout the country in order to be effective. A second area for concern is pollution control. Public Law (PL) 92-500 requires secondary sewage treatment for communities by 1977, industries by 1983, and some farms and feedlots beginning in 1978. Conventional aerobic liquid-waste treatment operations are energy-intensive and produce little fuel gas.

Fuel and energy conservation is potentially possible in wastewater treatment plants that utilize a new bioreactor called ANFLOW. The process consists of attaching microorganisms to special packing materials and passing liquid wastes upward through the unit for treatment. Using this system anaerobically, fuels and fuel gases are produced directly during waste treatment. There is also a potential reduction of the

operating energy requirements arising from the elimination of aeration required for aerobic processes and the lowering of sludge-handling requirements compared with those of conventional activated sludge treatment systems. This process has been under investigation at Oak Ridge National Laboratory (ORNL) for over two years.

The objective of this program is to evaluate the ANFLOW bioreactor for the treatment of domestic sewage and industrial wastes and investigate its potential by determining system operating parameters, including energy requirements, fuel gas production, capital investment, and the quality of waste treatment afforded. It is anticipated that the technology will be applicable to energy conservation in the near future.

It is expected that the ANFLOW process, when fully developed and applied, could provide secondary quality sewage treatment for domestic and industrial wastes, in addition to providing sufficient fuel gas to have a measurable impact on U.S. fuel gas supplies. The process would also provide an energy savings through its low operating requirement. A main advantage of immediate process development is that PL 92-500, because of its time limitations for pollution abatement, will help make the fuel gas and the energy conservation benefits available in the later 1970s to early 1980s.

It is anticipated that the results of this program will demonstrate the process feasibility of a new waste treatment system that will: (1) require significantly less energy than is now used to operate conventional (aerobic) systems and concomitant sludge-handling facilities, (2) produce a valuable by-product in the form of a useable fuel gas which can substitute for petroleum-derived products, (3) reduce the capital investment required to construct waste treatment facilities, and (4) produce a treated liquid stream that will meet the EPA secondary treatment standards.

The immediate goals of this program are threefold:

1. To evaluate the process at the demonstration pilot plant scale for the treatment of domestic sewage. This has first priority in 1977 since domestic sewage (a) is the largest single type of liquid waste and (b) is treated municipally in amounts sufficiently large to make fuel gas recovery attractive.
2. To evaluate the potential industrial applications for the process.
3. To continue the supportive development work necessary to achieve the full potential of the process.

The technical methods for achieving these goals are:

1. To design and construct a demonstration pilot plant for the treatment of liquid domestic wastes with the cooperation and assistance of a private industrial company and a municipal government.
2. To operate the demonstration pilot unit, initially use the city of Oak Ridge municipal sewage as a development tool for the assessment and optimization of operating parameters and determination of scale-up factors. The bioreactor residence dependency for a larger plant is being determined, and energy production and conservation over long-term operation are being evaluated.
3. To measure sludge formation rates.
4. To cooperate with a private industrial company in assessing the market for the process application to domestic and industrial waste treatment, and to determine appropriate system configurations for such markets.
5. To identify system response to toxic materials that might decrease system efficiency for scale-up purposes. A laboratory test unit is being used to study biomechanisms and microorganism adaptation for an expected variety of liquid wastes.

6. To study improved means for preparation, storage, and transportation of specially coated, stabilized packing materials.

5.1.1 Pilot plant development

The ANFLOW Demonstration Pilot Plant was designed to process ~ 20 m³/day (5000 gal/day) of municipal sewage, using an ANFLOW bioreactor. The packed tower is a cylindrical tank 1.5 m (5 ft) in diameter and 5.6 m (18.3 ft) high, containing 3.0 m (10.0 ft) of packing, or 5.6 m³ (200 ft³) of packed volume. The column is to be packed with 25-mm (1.0-in.) Raschig rings. The pilot plant is located at the Oak Ridge East Waste Treatment Plant and will use raw sewage as feed.

A grinder pump will macerate the solids in the sewage and pump the liquid into the bottom of the column. As the sewage flows upward, the microorganisms clinging to the packing will break down the complex organic compounds in the sewage into simpler substances. The liquid effluent will flow from the top of the column, over a weir, and then back into the pretreatment section of the treatment plant. Gaseous products of the process will come off the top of the column and, after sampling and metering, will be vented to the atmosphere.

Process variables will be monitored continuously. The off-gas from the column will be sampled and analyzed for methane, carbon dioxide, and hydrogen sulfide by a process gas chromatograph. The volume of off-gas will be measured by a wet-test meter. Automatic samplers will take samples of feed and effluent streams and store them for later analysis. The pH of the feed and effluent streams will be monitored continuously, as will the temperature at various points. The flow rate of the feed stream will be recorded and controlled.

Site development. The pilot plant is located at the Oak Ridge East Waste Treatment Plant, adjacent to the raw sewage inlet. A nearby building is used to house instrumentation and miscellaneous equipment. The city of Oak Ridge has constructed a concrete foundation for the column and its attendant platform. In addition, the city has rerouted a digester effluent line at the site which would have interfered with the pilot plant operation. Further site preparation will consist of upgrading the electrical supply at the site to provide more power, replacing corroded steel grid flooring, and installing locks to provide protection for the equipment which will be stored in the building.

Design and construction. ORNL Engineering has provided a design for a concrete pad to support the ANFLOW column and the adjacent platform. The pad has been completed with materials and labor supplied by the city of Oak Ridge. The ANFLOW column was designed by Norton Company in conjunction with ORNL Engineering and Experimental Engineering Section personnel. It was fabricated from fiberglass at Norton's plant in Akron, Ohio, and delivered to Oak Ridge the end of September. The bottom is a 45° cone with a flanged outlet, and the top is flat with a view port. There are nozzles through the tank wall for feed inlet and gas outlet, and two auxiliary nozzles are located near the top and bottom. There are thermocouple taps near the top and bottom of the packed section, a U-tube manometer tap at the top, and sampling taps at 0.3-m (1.0-ft) intervals vertically along the packed section. An overflow weir and collection trough in the top of the column are designed to remove effluent from the center of the tank. The effluent then flows out of the column by passing under a partition and over a baffle before flowing back to the sewage flume. The arrangement prevents air from entering the top of the column. Taps are provided in the

trough for a thermocouple, the effluent outlet, and two air bubbler lines which will be used for flow control. The column will be insulated with 4 in. of fiberglass. A 100-mm (4-in.) gate valve will be installed on the cone flange. All piping outside the building will be insulated and electrically traced to prevent freezing.

A platform providing access to the entire column height was designed with stairs for personnel carrying apparatus, and each level is well lighted.

The building that will house the instrumentation is currently heated by a 15-kW electric heater. A remotely located thermostat will be provided to enable better temperature control.

The electrical supply will be altered to increase the power available by installing a 200-A service drop cable to the instrument building.

Instrumentation. Thermocouples will be placed in the top and bottom of the column, in the outlet trough of the column, and in the feed stream. Temperatures will be recorded on a multipoint Honeywell Brown recorder, with a 0 to 100°F full range (-17.8 to 37.8°C). The pH of the feed and effluent streams will be measured by Markson Model 1888 combination pH electrodes connected to Markson Model 4404 pH meters. The pH will be recorded on a multipoint Honeywell Brown recorder, 0 to 10 mV full range. The off-gas volume will be monitored by an American Meter Model A117-1 wet-test meter, with an integrating capacity of 10 m³ (350 ft³).

The composition of the off-gas will be monitored at preset intervals by a Bendix process gas chromatograph system, consisting of a Model 007 analyzer, Model 7000 programmer, thermal conductivity detector, and a sample conditioning unit. This system will automatically sample the off-gas stream, condition the sample, inject it into the chromatographic column,

integrate the peaks, and record percent composition for methane, carbon dioxide, and hydrogen sulfide. Recorder output will be presented in a bar graph format. Other constituents will be detected by manually switching to a spectrum output. The chromatograph output will be recorded on a single-pen Honeywell Brown recorder, 0 to 10 mV.

Gas bubblers in conjunction with a Foxboro differential pressure transmitter will be used to detect the height of liquid between the partition and the weir in the trough which is in the top of the column. The transmitter signal will be made proportional to a flow rate range of 3.8 to 38.0 m³/day (1000 to 10,000 gal/day). The transmitter signal will be recorded on a Foxboro controller-recorder, which will in turn control a three-way diverting pneumatic control valve on the feed line. Since the capacity of the feed pump is greater than the feed requirements, the control valve will allow the desired amount of sewage to enter the column while diverting the remainder of the pump output back into the sewage stream.

Samples of the feed and effluent streams will be taken automatically by two Pro-Porta-Tech CG-110 liquid samplers. These pneumatically operated samplers will take 50-ml samples at preset intervals and discharge the samples into refrigerated bottles.

Special equipment. A Toran Model 12SG1 grinder pump will be the feed supply pump. This is a submersible centrifugal pump, developing 15 m (50 ft) of head at 0.11-m³/min (30-gal/min) flow. Special cutters at the pump suction grind solid materials into pea-size particles. The pump is rated for continuous service, and, in the event that the pump runs dry, it can run for several days with no liquid. A Kellogg-American Model B321UB air compressor will be used to supply instrument air. A Pall-

Trinity-Micro Model 10HA10000E instrument air dryer will be used to remove water and oil from the instrument air.

Instrument details of the ANFLOW Demonstration Pilot Plant are shown in Engr. Dwgs. P3D20017C001 B, P3E20017C002 B, P3E20017C003 A, and S3E20017B002 O.

5.1.2 Supporting research

During the transition quarter, one of the Norton alternatives for packing the ANFLOW unit was evaluated. Although this material had not been previously subjected to testing in an anaerobic unit for extended periods of time, it had been evaluated as a support for immobilized enzymes. In order to test the packing, an 8-in. (16-cm) diameter by 6-ft (1.8-m) high column was filled on June 24, 1976, with 1.8 ft³ (51 liters) of Norton HSA Raschig rings 0.5 in. (1.2 cm) in diameter (Sample No. 46358). Four gallons (15 liters) of rumen fluid and 6 gallons (23 liters) of sewage sludge were added to seed the unit. During operation, the column was fed continuously at a feed rate of 1 ml/sec using the feed mixture shown in Table 5.1. Throughout a several week period, the packing formed an even coating of microorganisms, and anaerobic protozoans were found throughout the bioreactor. Gas production, shown in Fig. 5.1, increased for several weeks. No large-scale deterioration of the packing material has been evident during the period of testing. However, this is not necessarily conclusive, since full-scale units may be expected to be in operation for a period of years, rather than weeks.

Drawings were prepared for the units to be used for hydraulic and organic overloading testing. The units are being completed, as scheduled, in the shop and will be ready for testing in early October.

Table 5.1. Feed composition

Compound	Concentration (mg/liter)
Sodium acetate	200
Lactose	200
Mannitol	200
Acetic acid	100
Butyric acid	30
Valeric acid	30
Propionic acid	30
Sodium bicarbonate	50
Magnesium sulfate	16
Sodium monohydrogen phosphate	200
Yeast extract	100
Tryptone	100

ORNL DWG 77-1634

Fig. 5.1. Gas production by ANFLOW unit using 1/2-in. HSA alumina rings.

5.2 Biotreatment of Coal Conversion Aqueous Effluents

Phenols, thiocyanates, and ammonia are the major types of contaminants to be removed from coal conversion aqueous waste streams. This is a continuation of previous reports¹⁻³ on biological degradation of phenol in a three-phase (liquid-gas-solid biomass catalyst) TFBBR. The reactor feed contained phenol, phenol and resorcinol, phenol and m-cresol, or phenol and atmospheric hydrocarbonization wastes (Table 5.2). Only phenol can be determined analytically without great difficulty and cost. Analytical methods used for phenols were the normal 4-aminoantipyrine assay, direct injection gas chromatography, derivitized gas chromatography, and direct-injection high-resolution liquid chromatography. Organic carbon content was analyzed using a Dohrmann Envirotech DC-50 carbon analyzer. Off-gas composition was determined by gas chromatography. Cell counts were done by plating and counting colonies of effluent samples from the reactor.

This report covers the tests of Phenobac breakdown of thiocyanate and Bethlehem Steel activated sludge breakdown of phenols and thiocyanates. The reactor used is the redesigned unit described in the last report. The seed bacteria were grown in a shake flask and then placed into the column and grown until visible. The anthracite coal of 25 to 35 mesh size was added, and the bacteria was allowed to attach themselves to the coal. During this time, the reactor was operated in total recycle mode, with phenol being added occasionally. After attachment of the bacteria, the reactor was operated on a once-through basis, with no recycle. Oxygen supply was facilitated by a pure oxygen sparge into the bottom of the column.

Table 5.2. Degradation rates in tapered fluidized-bed bioreactor - activated sludge

Column feed stream				Column e-fluent				Reactor conversion rates ^a				
Flow rate ^b (ml/min-cm ²)	Phenol (ppm)	Thiocyanate (ppm)	Soluble organic carbon (ppm)	Phenols (ppb)	Thiocyanate (ppm)	Soluble organics (ppm)	Biomass carbon (ppm)	Phenols degradation rate (g/day-l)	Thiocyanate degradation rate (g/day-l)	Soluble carbon degradation rate (g/day-l)	Biomass production of carbon rate (g/day-l)	CO ₂ production rate (l/day-l)
8.88 ^c	87	150	76	500	150	37	10	11.2	0	5.04	1.296	
10.64	184	150	136	76,000	150	98	-	16.76	0	5.89		
0.351 (12.94) ^d	1516 (41)	145 (14)	933 (109)	50	10	34	282	7.76	0.69	4.36	1.44	
0.366 (12.94)	1510 ^e (43)	135 (4.6)	925 (72)	410	0.75	46	284	8.04	0.71	4.69	1.515	0.571
0.387 (12.96)	1565 ^f (46.8)	172 (9.9)	955 (82)	140	4.8	52	340	8.84	0.93	5.33	1.922	0.591
0.358 (12.94)	700 ^g (19.4)	90 (3.5)	795 (259)	70	1.0	237	111	3.67	0.47	1.24	0.58	0.322
0.384 (12.96)	1050 ^g (31)	208 (8.9)	740 (165)	150	2.75	143	85	5.88	1.156	3.33	0.48	0.45

^aReactor conversion rate based on volume of reactor containing coal particles - 4.5 liters.^bFlow rate based on largest column cross-sectional area (3-in. ID section).^cFeed flow through column - no recycle.^dNumbers in parentheses refer to actual feed to column including recycle stream.^e50% phenol--50% resorcinol.^f50% phenol--50% m cresol.^gAtmospheric hydrocarbonizer aqueous waste + phenol + thiocyanate.

As previously reported,³ oxygen transfer from the gas phase to the liquid phase is the limiting step in phenol degradation by both Phenobac and activated sludge microorganisms. The reaction rate for the activated sludge is slightly lower, by $\sim 20\%$, than that for Phenobac.

The unit was operated for ~ 1 month using Phenobac to degrade varying concentrations of thiocyanate in the absence of phenol. The degradation rate was $\sim 10\%$ of the rate for phenol. Results of this test are shown in Table 5.3. In general, the rate was independent of the thiocyanate feed concentration from 50 to 200 ppm. Thiocyanate content was analyzed by the standard ferric nitrate colorimetric assay, with a lower detection limit of 1 ppm.

The unit was shut down, cleaned, and started up using the activated sludge seed bacteria. Initial operation was conducted with no recycle and with phenol feed concentrations from 25 to 220 ppm phenol. The degradation rates and associated data are shown in Table 5.2. The maximum phenol degradation rate was 16.84 g/day-liter, based on a 4.5-liter reaction zone in the tapered fluidized-bed reactor. No degradation of thiocyanate was noted, and total organic carbon reductions of 20 to 50% were observed. As shown in the table, the maximum rates occurred when there was a significant level of phenol in the reactor effluent. When low phenol effluent levels were obtained (< 1 ppm), the degradation rate was lower (a maximum of 11.11 g/day-liter).

The second test was conducted using the reactor in a recycle mode, with the concentrated feed (700 to 2000 ppm phenol) diluted by the recycle stream. In this mode, the maximum rate obtained with a phenol effluent level < 1 ppm was 8.45 g/day-liter phenol degraded. After 2 weeks of operating in this mode, thiocyanate began to be degraded. The results

Table 5.3. Thiocyanate degradation rates in a tapered fluidized-bed bioreactor^a

Feed stream		Effluent	
Flow rate (ml/min)	CNS ⁻ concentration (ppm)	CNS ⁻ concentration ^b (ppm)	conversion rate ^c (g/day-liter)
70	158	110	1.08
75	170	106	1.54
52	95	34	1.02
64	102	36	1.35
30	82	8	0.71
73	50	6	1.03
83	50	2	1.27

^aAll runs were made at ambient pressure, $25 \pm 2^\circ\text{C}$, pH 7.2 to 7.6, using Phénobac.

^bAssay was sensitive to 1 ppm.

^cIncluded the volume of the fluidized bed, as well as solution above the bed (4.5 liters).

are shown in Table 5.2 for the feed compositions noted. During this test, samples were analyzed for total carbon, and the off-gas was analyzed for CO_2 in an attempt to assess the carbon balance over the reactor. The results show that in this recycle mode (recycle with phenol feed), 60 to 65% of the feed carbon was converted to CO_2 , 30% was converted to biomass carbon, and 5 to 10% remained as soluble carbon in the effluent stream. Up to 6% of the off-gas stream was composed of CO_2 under some conditions.

One of the major problems encountered was the appearance of mold contamination in the reactor. During operation using a feed containing atmospheric hydrocarbonizer waste from runs 3 and 4 (August 1975) stored in the cold room for almost 1 year, mold growth in the reactor developed. The mold overwhelmed the bacteria and coated the reactor walls and coal particles, sometimes to a thickness of several millimeters. The growth took place in 2 to 3 days, after which the reactor and coal were cleaned and returned to operation. However, growth again developed, and after several recurrences, the column, coal, and feed tank were thoroughly cleaned, and phenol feed without the atmospheric hydrocarbonizer waste was reinstituted. The column was then seeded with bacteria. Mold growth was no longer observed.

When changing the feed composition (i.e., from phenol to phenol and resorcinol) a lag period of ~ 12 hr was observed before the new substrate was efficiently degraded. After this lag, the degradation rate returned to approximately the same rate (before the change). Thiocyanate continued to be degraded efficiently during these changes as long as very low effluent phenol levels were maintained. When higher effluent phenol levels were observed, thiocyanate degradation efficiency fell.

During operation with the coal atmospheric hydrocarbonizer waste, samples were analyzed on a HRLC and by direct injection gas chromatography and the original peaks contained in the feed stream were not detectable. For example, phenol and catechol were detected in the feed but not in the effluent.

Tables 5.2 and 5.3 show results using Phenobac for the degradation of thiocyanate and the Bethlehem Steel activated sludge experiments.

Also, during this reporting period, a facility was designed and constructed in Building 2528 for treatment of the aqueous waste produced by the bench-scale coal hydrocarbonizer (see Fig. 5.2).

5.3 ORNL Support of the Nitrate Waste Recycle Facility at Y-12

The biotechnology development staff of EES has maintained a continuous interest and effort in biodenitrification, as well as periodic interaction with J. M. Napier and his staff of the Y-12 Development Division, who are responsible for the design, installation, and start-up of the "Nitrate Waste Recycle Facility" at Y-12.

It was agreed that the EES biotechnology staff would assist the Y-12 biodenitrification group with several specific tasks: (1) backup storage of seed bacteria, (2) bacterial growth during reactor start-up, and (3) determinations of uranium and plutonium balance in bioreactor sludge.

5.3.1 Task I - backup storage of seed bacteria

The seed bacteria received from F. Clark on 3/22/76 were frozen and stored in Buildings 4500N and 4505. On 5/13/76 and 9/17/76 (52 and 180 days after freezing), a sample from each freezer was thawed and plated for colony count (see Table).

Fig. 5.2. Tapered fluidized-bed bioreactor for the treatment of aqueous wastes from coal conversion processes.

	Date		
	3/22/76	5/13/76	9/17/76
Freezer at -60°F, Building 4500N (colony/ml)	8×10^9	$2 \times 10^{+10}$	$1 \times 10^{+10}$
Freezer at -10°F, Building 4505 (colony/ml)	8×10^9	5×10^9	$3 \times 10^{+8}$

The automatic defrost failed to function correctly in Building 4505 and allowed these samples to thaw and refreeze a number of times during the interval between 5/13/76 and 9/17/76.

5.3.2 Task II - bacterial growth during reactor start-up

Four very successful start-up tests have been completed using a 16-gal bench-scale bioreactor from Y-12 Development. "An Operational Guide to the Y-12 Denitrification Facility," by J. M. Napier, Y-DA-6720, was used as a guideline for the test.

Test 1 did not have continuous pH control (20% NaOH was used for pH adjustment for all tests), a necessity for good operation of this size test reactor. Sudden pH changes cause chemical shock to the bacteria, which results in a slowing of the growth rate. The denitrification bioreactor start-up feed solution should have a carbon/nitrogen ratio sufficiently large so that the bacteria will not consume all of the carbon (as acetate) before depletion of nitrates; the stoichiometric carbon/nitrogen ratio is $\sim 1.2/1.0$. Carbon starvation can cause a growth shock and should be avoided. A carbon/nitrogen ratio of 2.1/2.5 was used for tests 2 and 3, which showed higher nitrate conversion rates sooner than test 1. The data are shown in Table 5.4.

Table 5.4. Results of four denitrification start-up tests
16-gal bioreactor $\approx$ 57 liter MINI XII

	Test 1	Test 2	Test 3	Test 4
Start-up colony count, colony/ml	$1 \times 10^{+7}$	$1 \times 10^{+7}$	$1 \times 10^{+6}$	$8 \times 10^{+7}$
Colony count after 10 days, colony/ml	$2 \times 10^{+8}$	$6 \times 10^{+8}$	$4 \times 10^{+9}$	$2 \times 10^{+9}$
Start-up carbon/nitrogen ratio	1.2	2.1	2.5	2.1
pH control	no	yes	yes	yes
Nitrate removal rate (g/day-liter per day after start)	2.0/14	4.0/14	6.0/9	4.0/16

Table 5.5. Uranium solubility in centrifuged aqueous
phase during denitrification (Test No. 4)

Test day	Feed uranium concentration (ppm)	Tank uranium concentration (ppm)	Bacteria population (cells/ml)	Denitrification rate g NO ₃ /day-liter
13			$2 \times 10^{+9}$	3
16	-/100		$3 \times 10^{+9}$	4
19	100/250	38	$5 \times 10^{+9}$	4
26	250/500	69	$9 \times 10^{+9}$	5
28	500	179	$6 \times 10^{+9}$	6

The purpose of test 3 was to use 10% of the normal seed bacteria at start-up and follow the growth data. The initial nitrate decrease period was determined by an off-gas rate increase and frequent on the spot nitrate assays. The feed was started before the nitrate was completely removed, thus eliminating a nitrate starvation shock from which the bacteria are slow to recover.

On day 11 of test 3, chromium as $\text{Cr}(\text{NO}_3)_3$ was added to the feed at a level of 80 ppm chromium with no effect on the system. The chromium level in the reactor was < 0.1 ppm in the centrifuged supernate on day 17. The Cr^{+3} in the feed was increased to 230 ppm on day 17 and to 430 ppm on day 19. The test was terminated on day 23. At that time, the Cr^{+3} concentration would have been ~ 100 ppm in the tank. However, due to the Ca-Al-CO_3 precipitation properties, the chromium was removed from soluble portions of the reactor contents to a level of < 0.1 ppm (Cr^{+3}). The reactor's ability to remove Cr^{+3} , $\text{Fe}^{+2,3}$, and PO_4^{-3} ions as precipitates exhibited the need to add micronutrients daily for bacterial growth.

Test 4 was conducted under the same chemical and bacterial conditions as test 2, except that depleted uranium was added in massive quantities to determine its effect on bacterial growth and the uranium solubility in the presence of the Ca-Al-CO_3 precipitate (Table 5.5). This test took longer to reach a satisfactory denitrification level because of mechanical problems (pump failure, etc.). The denitrification rate was 4 g/day-liter after 16 days, and the bacteria count was $3 \times 10^{+9}$ cells/ml, at which time depleted uranium was added, thus increasing the tank and feed concentration to 100 ppm uranium.

On day 19, the denitrification rate was still 4 g NO_3 /day-liter, and bacteria population had increased slightly to $5 \times 10^{+9}$ cells/ml. No serious effect of 100 ppm uranium feeding was noted. The uranium solubility was 38 ppm.

The uranium concentration was then raised to 250 ppm in the feed and tank. Seven days later (day 26), the uranium concentration in the tank was 69 ppm, the bacteria population was $9 \times 10^{+9}$ cells/ml, and the denitrification rate was 5 g NO_3 /day-liter. The uranium concentration in the feed and tank was increased to 500 ppm for the remainder of the test. On the last day of the test (day 28), the denitrification rate was 6 g NO_3 /day-liter, the uranium solubility was 179 ppm, and the bacteria population was $6 \times 10^{+9}$ cells/ml. Uranium concentrations up to 500 ppm apparently have no adverse effect on denitrification rates or bacteria population. However, the solubility of uranium is much greater than other metal ions such as iron and chromium because of the high solubility of uranium carbonate.

5.3.3 Task III - determination of uranium and plutonium balance in bioreactor sludge

During the test 3 study of bacterial growth in the 16-gal bench-scale bioreactor, samples were analyzed to determine uranium, plutonium, and chromium deposition in the reactor sludge. Plutonium levels in all samples were below the Y-12 Analytical Lab detection limit of 1.3×10^{-5} $\mu\text{Ci/g}$.

Test results shown in Table 5.6 indicate that for a uranium level of 30 ppb in the feed, 67 to 94% of the uranium was removed by sludge and "free cells." Furthermore, increases in the chromium level in the feed from 30 to 1030 ppm had no deleterious effect of uranium removal.

Table 5.6. Uranium and chromium uptake by reactor sludge and free cells during Test 3. Uranium concentration in the feed was 30 ppb

	Test day 10	Test day 25	Test day 12
Uranium concentration in feed, ppm	30	430	1030
Dissolved chromium in reactor, ppm	<0.1	<0.1	<0.1
Dissolved uranium in reactor, ppb	11	2	4
Dissolved chromium removal, %	>99	>99	>99
Dissolved uranium removal, %	67	94	88
Chromium distribution coefficient for sludge	>1400 ^a	>9110	>2620
Uranium distribution coefficient for sludge	18 ^a	115	20
Chromium distribution coefficient for free cells	a	35	b
Uranium distribution coefficient for free cells	a	16	69

^aSludge and free cells analyzed as composite sample.

^bChromium concentration on free cells below detection limit of 0.1 ppm.

A similar study was made during test 4 wherein depleted uranium was added to the bioreactor. Again, samples were analyzed to determine uranium and chromium deposition in the reactor sludge. The results are given in Table 5.7. As shown previously, uranium concentration up to 500 ppm in the feed apparently had no adverse effect on the denitrification rate or cell population.

Table 5.7 Uranium and chromium uptake by reactor sludge and free cells during Test 4. Chromium concentration in the feed was 30 ppm

	Test day 19	Test day 26	Test day 28
Uranium concentration in feed, ppm	100	250	500
Dissolved uranium in reactor, ppm	38	69	179
Dissolved chromium in reactor, ppm	<1	<1	<1
Dissolved uranium removed, %	62	72	64
Dissolved chromium removal, %	>97	>97	>97
Uranium distribution coefficient for sludge	12	12	4
Chromium distribution coefficient for sludge	>24	>25	>16
Uranium distribution coefficient for free cells	2	1	1
Chromium distribution coefficient for free cells	a	a	a

^aIndeterminant since dissolved chromium was below detection limit.

5.4 Tapered Fluidized-Bed Bioreactor Studies

5.4.1 Ethanol production

This test system was designed to demonstrate the use of a TFBBR as a tool to produce a valuable product, in comparison with its previous use as a process to remove or alter a potentially harmful chemical substrate such as phenol or nitrate. The production of ethanol for fuel use is a realistic ERDA goal.

The bacteria species *Zymomonas mobilis* (*Z. mobilis*) ATCC No. 10988, a type of *Pseudomonas* bacteria, was chosen because its yield of ethanol is greater than that of *Lactobacillus* or any other common bacterial strain.

The *Z. mobilis* was grown in yeast and/or pantothenic acid media (Table 5.8). An active growing and gassing bacteria culture was introduced into a -30 +60 mesh (500-ml settled bed volume) bed of fluidized coal and growth media and was recycled for a number of days to allow the bacteria to attach to the solid (coal) fluidizing support. The glucose was assayed daily using a glucose "Stat pack". The stoichiometric ratio of 1.7 moles of ethanol per mole of glucose was checked experimentally and was found to be valid. The glucose assay was used for the remainder of the testing period.

When the bioreactor started release of CO₂ gas and the glucose disappearance rate was measurable, the bioreactor was tested in a number of operating modes: total recycle, low feed-to-recycle volume ratio, and straight-through feed. The bacteria did not attach themselves tightly to the coal; a large amount of free-floating bacteria was observed.

Table 5.8. Feed for *Zymomonas mobilis*^a

	Concentration (g/liter)
Glucose	100
Yeast extract	5
Peptone	5
KH ₂ PO ₄	0.1
NH ₄ Cl	10
Trace metal	1 ml (std mix)
pH	7.5 (NaOH)
Panothentic acid	10 ⁻⁶ moles
(to replace yeast extract and peptone)	

^aJ. P. Belakh, A. Belakh, and P. Simon,
Petri. J. of Gen. Mico. 70, 179-85 (1972).

A typical recycle test used two TFBBRs in series, with a total settled volume of coal support of 1000 ml. The recycle rate was 370 ml/min, while the 100-g/liter glucose feed rate was 14 ml/min. The mixed feed and recycle glucose concentration was 65 g/liter, and the glucose concentration leaving the bioreactor was 63 g/liters--a reduction in glucose concentration of 2 g/liter, which calculated to be a conversion rate of 226 g/day-liter based on an expanded bed volume of 3.3 liters.

Results from this test point out that the operating conditions used were less than optimum. Conditions should be chosen so that the volumetric flow rate for particle fluidization is consistent with a residence time in the reactor for sufficient conversion of reactant. System parameters such as particle size, particle density, and reactor geometry can be chosen for optimal reactor performance.

A very tall TFBBR was constructed and tested for ethanol production. The bioreactor was 13 ft high. It consisted of a bottom section 9 ft long

and 1 in. in diameter, followed by a tapered section 2 ft long and 1 to 2 in. in diameter, and above that a solid disengaging section (Fig. 5.3) 2 ft long and 2 in. in diameter. The expanded bed volume is 1600 ml at the normal operating level of 1 ft in the tapered section.

The following recycle test was performed to evaluate the tall bioreactor system. The bioreactor was operated using a 109-g/liter glucose feed (Table 5.8). The feed was pumped at the fluidizing rate of 100 ml/min for 24 hr to establish the steady state operating conditions corresponding to a glucose consumption rate of 270 g glucose/day-liter. Then at the start of recycle part of the test, the bioreactor discharge line was placed in a 1-liter mixed tank filled with feed. The mixed feed tank contents were fed in a recycle mode to the bioreactor for the next 10 hr. The discharge stream was sampled prior to the mixed feed tank each hour; the glucose concentration vs time is shown in Fig. 5.4.

The glucose consumption rate was 273 g glucose/day-liter based on a 1600-ml expanded bed volume. The straight line part of the plot of decreasing glucose concentration vs time indicated a zero-order reaction rate (w.r.t. glucose), with no inhibition due to reaction products. However, as the substrate concentration decreased and/or the by-products increased, a rate inhibition appeared (as shown by the curved section of the later part of the test).

The *Z. mobilis* test of ethanol production from glucose feed was successful. The glucose consumption rate was nearly constant from 50- to 300-g/liter glucose. The *Z. mobilis* bacterial system was not invaded by any other microorganism (mold, yeast, other bacteria) during the 90 days of nonsterile operation.

Fig. 5.3. Tapered fluidized-bed bioreactor (1-in. diameter) for ethanol fermentation.

Fig. 5.4. Reduction of glucose as a function of time in a tapered fluidized-bed bioreactor (1-in. diameter).

5.4.2 Variable Angle Test Unit

A Variable Angle Test Unit (VATU) was constructed at ORNL from 1/2-in. thick Plexiglas, as shown in Fig. 5.5. The VATU has movable side walls that form a rectangular channel 2.54 by 5.08 by 114.3 cm when the angle is adjusted to zero (Table 5.9). The channel height is constant because the side walls are longer than the 114.3-cm-high overflow pipe. The side walls can be moved to provide a tapered channel with half angles of 0, 0.32°, 0.72°, 1.39°, and 1.94°.

Table 5.9. Variable Angle Test Unit dimensions

Length	Channel size (cm)		Angle (half)	Volume (cm ³)
	Top width	Bottom width		
114.3	10.2	2.54	1.94°	3.85
114.3	8.1	2.54	1.39°	3.17
114.3	5.4	2.54	0.72°	2.38
114.3	3.8	2.54	0.32°	1.90
114.3	2.54	2.54	0.00°	1.48

A test was performed using 500 g of -25 +40 mesh coal at flow rates from 0 to 1200 ml/min for each of five taper angles. A plot of hydraulic pressure drop, expanded bed height, and void volume vs water flow from 0 to 1200 ml/min is shown in Fig. 5.6. This unit will be used to evaluate the effect of the taper angle on the hydraulic and chemical performance of the unit. These are preliminary data to be followed by further tests such as a dispersion study.

Fig. 5.5. Variable Angle Test Unit.

Fig. 5.6. Hydraulic pressure drop, expanded bed height, and void volume vs water flow from 0 to 1200 ml/min.

5.5 References for Section 5

1. C. D. Scott et al., Experimental Engineering Sect. Semiannu. Progr. Rep. (Excluding Reactor Program) for the Period Sept. 1, 1974 through Feb. 28, 1975, ORNL/TM-4961 (January 1976).
2. C. D. Scott et al., Experimental Engineering Sect. Semiannu. Progr. Rep. (Excluding Reactor Program) for the Period Mar. 1, 1975 through Aug. 31, 1975, ORNL/TM-5291 (September 1976).
3. C. D. Scott et al., Experimental Engineering Sect. Semiannu. Progr. Rep. (Excluding Reactor Program) for the Period Sept. 1, 1975 through Feb. 29, 1976, ORNL/TM-5533 (in press).

THIS PAGE
WAS INTENTIONALLY
LEFT BLANK

INTERNAL DISTRIBUTION

ORNL/TM-5865/V2

1. Biology Library	51. V. A. Jacobs
2-3. ORNL - Y-12 Technical Library	52. W. F. Johnson
4-5. Central Research Library	53. R. L. Jolley
6. Document Reference Section	54. O. L. Keller
7-9. Laboratory Records	55. J. A. Klein
10. Laboratory Records-RC	56. D. D. Lee
11. ORNL Patent Section	57. N. E. Lee
12. MIT Practice School	58. R. E. Leuze
13. S. I. Auerbach	59. A. P. Malinauskas
14. J. A. Auxier	60. J. S. Mattice
15. R. E. Barker	61. L. E. McNeese
16. M. L. Bauer	62. D. L. Million
17. J. T. Bell	63-65. J. E. Mrochek
18. W. D. Bostick	66. E. Newman
19. R. W. Brocksen	67. G. E. Oswald
20. R. E. Brooksbank	68-70. W. W. Pitt
21. C. H. Brown	71. H. Postma
22. K. B. Brown	72. C. R. Richmond
23. W. D. Burch	73. B. R. Rodgers
24. R. M. Canon	74. M. W. Rosenthal
25. S. D. Clinton	75. D. D. Schuresko
26. H. D. Cochran	76-77. C. D. Scott
27. C. F. Coleman	78. W. D. Shults
28. A. L. Compere	79. S. E. Shumate
29. R. B. Cumming	80. M. G. Stewart
30. V. A. DeCarlo	81. J. B. Storer
31. M. S. Denton	82. G. W. Strandberg
32. S. R. Dinsmore	83. I. L. Thomas
33. B. Z. Egan	84. J. B. Talbot
34. D. E. Ferguson	85. D. B. Trauger
35. L. M. Ferris	86. R. I. Van Hook
36. P. W. Fisher	87. J. S. Watson
37. W. Fulkerson	88. J. R. Weir
38. R. K. Genung	89. P. R. Westmoreland
39. R. W. Glass	90. D. D. Willis
40. W. L. Griffith	91. R. G. Wymer
41. C. W. Hancher	92. E. L. Youngblood
42-46. J. R. Hightower	93. A. Zucker
47. H. N. Hill	94. G. R. Choppin (consultant)
48. R. M. Hill	95. E. L. Gaden (consultant)
49. D. W. Holladay	96. C. H. Ice (consultant)
50. H. W. Hsu	97. L. E. Swabb
	98. K. D. Timmerhaus

EXTERNAL DISTRIBUTION

99. C. E. Carter, Division of Biomedical and Environmental Research, DOE, Washington, D.C. 20545
100. C. W. Edington, Division of Biomedical and Environmental Research, DOE, Washington, D.C. 20545
101. R. P. Epple, Division of Physical Research, DOE, Washington, D.C. 20545
102. W. J. Haubach, Division of Physical Research, DOE, Washington, D.C. 20545
103. Carl Larson, Office of Life Sciences, NASA Headquarters, 600 Independence Avenue, Washington, D.C. 20546
104. Carolyn Leach, Mail Code DB-6, National Aeronautics and Space Administration, Houston, Texas 77058
105. J. L. Liverman, Division of Biomedical and Environmental Research, DOE, Washington, D.C. 20545
106. H. V. Malling, Environmental Mutagenesis Branch, National Institute of Environmental Health Sciences, Research Triangle Park, North Carolina 27709
107. C. I. Mashni, Environmental Research Center, U.S. Environmental Protection Agency, Cincinnati, Ohio 45268
108. Robert S. Melville, Research Grants Branch, National Institute of General Medical Sciences, NIH, Bethesda, Md. 20014
109. W. E. Mott, Division of Environmental Control Technology, DOE, Washington, D.C. 20545
110. C. L. Osterberg, Division of Biomedical and Environmental Research, DOE, Washington, D.C. 20545
111. E. S. Pierce, Division of Physical Research, DOE, Washington, D.C. 20545
112. F. Dee Stevenson, Division of Physical Research, DOE, Washington, D.C. 20545
113. R. W. Wood, Division of Biomedical and Environmental Research, DOE, Washington, D.C. 20545
114. Research and Technical Support Division, DOE, Oak Ridge Operations Office, P. O. Box E, Oak Ridge, Tenn. 37830
- 115-116. Technical Information Center, Oak Ridge, Tenn. 37830